DEMSEN

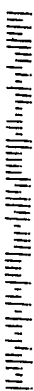TC1600
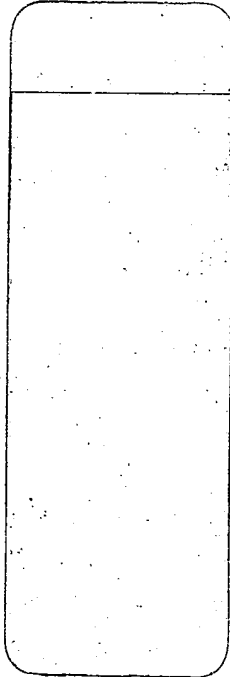
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UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/031,410	06/21/2002	Peter Eriksson	59760 (47137)	2145

21874	7590	03/03/2006

EDWARDS & ANGELL, LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER
MCGILLEM, LAURA L

ART UNIT	PAPER NUMBER
1636	

DATE MAILED: 03/03/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 10/03)

	Application No.	Applicant(s)
Office Action Summary	10/031,410	ERIKSSON ET AL.
	Examiner	Art Unit	
	Laura McGillem	1636	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _3_ MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
  Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on _07 February 2006_.

2a)☐ This action is **FINAL.**   2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle,* 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) _1-6,8-29 and 31-33_ is/are pending in the application.

   4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) _1-6,8-29 and 31-33_ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

   Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

   Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☒ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   a)☒ All b)☐ Some * c)☐ None of:

   1.☒ Certified copies of the priority documents have been received.

   2.☐ Certified copies of the priority documents have been received in Application No. _____.

   3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

   * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
   Paper No(s)/Mail Date _____.
4) ☒ Interview Summary (PTO-413)
   Paper No(s)/Mail Date. _2/7/06, 2/24/06_ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

U.S. Patent and Trademark Office

PTOL-326 (Rev. 7-05)   Office Action Summary   Part of Paper No./Mail Date 02242006

DETAILED ACTION

It is noted that the After Final amendment filed 2/7/06 has been entered and will be considered. Claim 1 has been amended, and claims 30 and 34 have been cancelled.

Finality of the previous Office Action is withdrawn in view of the newly discovered reference(s) to Magae et al (Appl. Micro. Biotechol., 1986, Vol. 24, 509-511) and Pui et al) U.S. Patent No. 6,093,557. Rejections based on the newly cited reference(s) follow.

Claims 1-6, 8-29 and 31-33 are under examination.

Oath/Declaration

The oath or declaration is defective. A new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. See MPEP §§ 602.01 and 602.02.

> The oath or declaration is defective because:
> Non-initialed and/or non-dated alterations have been made to the oath or declaration. See 37 CFR 1.52(c).

Inventor Moscho has made changes to his post office address, which do not appear to be initialed.

Claim Objections

Claim 5 is objected to because of the following informalities: There is a period after the word "electrical". Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-6, 8-29 and 31-33 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for *in vitro* selective electrofusion of at least two fusion partners having cell-like membranes, does not reasonably provide enablement for *in vivo* electrofusion of two fusion partners, or for conducting *in vitro* fertilization by selective electrofusion of an egg cell or an enucleated egg cell, and a sperm cell at any development stage, or for conducting non-human cloning. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims.

The test of enablement is whether one skilled in the art could make and use the claimed invention from the disclosures in the application coupled with information known in the art without undue experimentation *United States v. Telectronics*, Inc., 8 USPQ2d 1217 (Fed. Cir. 1988). Whether undue experimentation is required is not based upon a single factor, but rather is a conclusion reached by weighing many factors. These factors were outlined in *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Inter. 1986) and again in *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988) and include the following:

1) Scope of the claims. The claims are drawn to methods of selective electrofusion of at least two fusion partners having cell-like membranes using an electric

field of a strength sufficient to obtain fusion provided by at least one microelectrode that is sufficiently small to permit selective fusion. The claims encompass any combination of at least two fusion partners having cell-like membranes including cells, liposomes, proteoliposomes, synthetic vesicles, egg cells, enucleated egg cells, sperm cells at any development stage and plant protoplasts. The claims encompass *in vitro* fertilization using egg cells, enucleated egg cells, and sperm cells at any development stage from any species. The claims encompass cloning, excluding human cloning, of any other species besides humans. The claims are drawn to *in vivo* selective electrofusion including administration of pharmaceutically active substances to a cell or to a tumor, which encompasses a very large group of undisclosed pharmaceutically active substances and fusion partners as well as a large group of possible *in vivo* cells or tumors. The scope of the claims is broad and far reaching. The specification does not disclose or contemplate the use of egg cells, enucleated egg cells or sperm cells at any development stage for any use other than *in vitro* fertilization or non-human cloning.

2) State of the Art. A recent review by Sakai et al (Birth Defects Res. C Embryo Today 2005., Vol. 75. No. 2. pp.151-62) teaches that several mammalian species have been cloned by somatic cell nuclear transfer, but that the short and long term effects of cloning and assisted reproductive techniques are largely unknown (see page 152, left column, 1st paragraph). Sakai et al teach that systematic studies of cloned offspring are necessary, but are complicated by the low proportion of live offspring from nuclear transfer eggs, which is currently 2-3% regardless of species or nuclear transfer technique (see page 152, center column, 1st full paragraph). Sakai et al

teach that studies of cloning and cloned animals are limited by the ability to generate a

sufficient number of age-matched cloned animals and difficulty in designating

appropriate controls (see page 152, right column, 1st paragraph). Sakai et al teach that

reasons for low efficiency of cloning are currently unclear, but that exposure of eggs and

embryos to *in vitro* culture conditions, such as culture medium that may contain

chemicals at non-physiological concentrations, can affect embryonic development and

result in offspring abnormalities (see page 159, right column, 1st full paragraph, for

example). Sakai et al teach that although *in vitro* fertilization techniques are widely

used in livestock production, *in vitro* embryo culture has been associated with abnormal

physiology and morphological development and a perinatal mortality at a higher rate

than natural fertilization. Niemann and Rath (Theriogenology, 2001 Vol. 56. No.8.

abstract) teach that there are differences in the progress of *in vitro* reproductive

techniques among livestock such as cattle, sheep and swine. Sakai et al suggests that

the amount of cellular trauma and damage to eggs, sperm and embryos during

manipulation could result in negatively impacted cellular development and may depend

on the technical skill of the individual manipulating the gametes and embryos (see page

160, left column, 1st paragraph, for example).

Orentas et al (Cell Immunol. 2001., Vol. 213, No. 1, pp 4-13) teach that cellular

fusion can be used to generate anti-tumor immunity treatments such as by fusion of

dendritic cells containing tumor-derived material with other cell types. Orentas et al

disclose that such fusions have previously been performed using polyethylene glycol

(PEG) but that electrofusion is superior to PEG-mediated fusion and may be useful to

produce more potent cancer vaccines (see page 4, right column, 3rd paragraph and page 12, left column, 4th paragraph, for example). Mekid and Mir (Biochim Biophys Acta. 2000. Vol. 1524. No. 2-3, pp.118-30) teach that electropulses are used in electrochemotherapy to introduce drugs into tumor cells and DNA electrotransfer for gene therapy. Mekid and Mir found unexpected electrofusion of tumor cells as a result of electropulse methods and variability in fusion among different tumor cell types (see page 129, left column). Mekid and Mir suggest that the potential for electrofusion of tumor cells from electropulses, as well as introduction of cytotoxic drugs by electrochemotherapy or DNA electrotransfer could convey a therapeutic advantage by increasing the chance of cell death even in cells which did not receive lethal doses of therapeutic drugs or genes (see page 129, left column, 1st and 2nd paragraph).

3) Unpredictability of the art.   The unpredictability of using methods of *in vitro* fertilization and cloning is manifested in multiple issues. Sakai et al teach that cloning methods and studies of cloning outcomes are hampered by the very low rate of clones produced and the difficulty of establishing appropriate controls. Sakai et al preliminarily conclude that clones are not always phenotypically identical to the somatic cell donors and that the cloned progeny often have adverse health conditions, such as increased body weight and advanced aging. Sakai et al teach that cloning technique is "still unpredictable" and requires comprehensive and systematic longitudinal studies of cloned animals (see page 152, right column, 1st paragraph). Sakai et al disclose that pre- and perinatal death rates in clones are significantly higher than controls regardless of species and that the reason for low efficiency of somatic cell cloning are currently

unclear (see page 153, center column, 1st paragraph).   Sakai et al teach that the variety

of findings in cloned animals of multiple species suggests that cloning has different

consequences among different species and even within a species.

Niemann and Rath (Theriogenology, 2001 Vol. 56. No.8. abstract) teach that

success rates for *in vitro* fertilization of porcine embryos is much lower than that of

cattle. Niemann and Rath teach that main problems for *in vitro* fertilization for swine

include insufficient cytoplasmic maturation of oocytes, low proportion of blastocysts and

high proportion of polyspermic fertilization (see abstract). Therefore, the unpredictability

being able to use the claimed methods of cloning and *in vitro* fertilization is based on

art-recognized unpredictability of similar methods even between species and that the

specification has not taught how to use the claimed methods to successfully conduct

cloning or *in vitro* fertilization.

Mekid and Mir (Biochim Biophys Acta. 2000. Vol. 1524. No. 2-3, pp.118-30)

teach variability in results from *in vivo* cell electrofusion. Mekid and Mir treated mouse

B16 melanoma and LPB sarcoma tumors and mouse liver with electrical pulses in order

to electropermeabilize cells (see page 119, right column, 1st paragraph). Mekid and Mir

teach that the effect of the electrofusion was tissue type-dependent. The B16 melanoma

tumor cells fused and formed syncytial areas and giant cells, but using the same

electropulse method and the same electrode, the LPB sarcoma cells did not produce

the same results as the B16 cells, even at higher voltages (see page 124, right column

and page 125, left column, for example). Mouse liver was also subjected to the same

electropulse as the tumor cells and did not result in electrofusion of the tumor cells,

even after an increase in voltage (page 125, right column, for example). Mekid and Mir suggest that the difference in electrofusion ability between tumor types and normal tissue is related to the presence of proteases in the interstitial fluid which might have reduced the surrounding extracellular matrix to bring the adjacent cells in closer contact (see page 128, right column, last paragraph). One of skill in the art would also recognize that such *in vivo* electrofusion methods (i.e. multiple liposomes containing a pharmaceutical fused with an *in vivo* cell or a network of cells) might result in uneven fusion among adjacent cells wherein one cell may fuse with many liposomes, but an adjacent cell may fuse with only one liposome or none at all. Therefore, the success of the claimed methods drawn to *in vivo* electrofusion and treatment of tumors or introduction of pharmaceuticals is unpredictable, because not all tumor cell types are subject to equal fusion using similar methods.

4) Amount of guidance provided. The specification has provided some guidance regarding the specific number, strength and duration of fusion pulses required to fuse several well-known cell types *in vitro*. The specification has provided some guidance regarding the size of a hollow electrode, including the dimensions of a specific electrode used to fuse two cells *in vitro*. The specification provides broad ranges for the electrical field strength necessary for fusion (i.e. 0.1-10 kV/cm) for pulses of 10 μs to several seconds in duration. The specification discloses that for method using multiple pulses a repetition rate of ~1 Hz should be suitable. The specification discloses that the length and strength of the pulses depend on the size of the partners to be fused. The specification provides preferable size ranges for the microelectrodes

such as an outer diameter of a few nm to ~100 μm, preferably 5-30 μm and most preferably 20 μm. The Applicants have not provided any guidance regarding the specific size and number of electrodes to be used for *in vivo* electrofusion, non-human cloning or *in vitro* fertilization. Applicants have not provided any guidance regarding the specific number, strength and duration of fusion pulses to be used for *in vivo* electrofusion, non-human cloning or *in vitro* fertilization. Applicants have not provided any guidance regarding the electrical field to be used for *in vivo* electrofusion, non-human cloning or *in vitro* fertilization. Applicants have not provided any guidance regarding differences in the claimed fusion method for any other cells beside those exemplified regarding potential variations related to cell type, age and growth conditions, especially for non-human cloning and *in vitro* fertilization. Applicants have not provided any information concerning any variations in the claimed method for various pharmaceutical agents to be delivered to cells or tumors. Therefore the Applicants have not provided guidance to perform the full scope of the claimed method without undue trial and error experimentation.

5) Working examples. Applicants have provided an example of cell-cell fusion of PC12 cells *in vitro* with information regarding specific number, strength and duration of fusion pulses. Applicants disclose but do not show: fusion of NG108 cells in a network, fusion of NG108 cells, Jurkat cells and COS7 cells, and fusion of NG108 cells to PC12 cell to create hybrid cells. Applicants have provided an example of cell-single vesicle fusion in which the cells have been pretreated with a protease, and do not provide information regarding specific number, strength and duration of fusion pulses.

Applicants have provided a third example in which NG108 cells are fused using

electrolyte-filled silica capillary electrodes which are defined by their dimensions and

include information regarding specific number, strength and duration of fusion pulses

required to fuse the cells. Applicants have not provided any example of non-human

cloning, *in vitro* fertilization of any organism or electrofusion to deliver pharmaceutically

active agents *in vivo* or for tumor treatment.

6) Nature of the invention. The nature of the invention is drawn to in vivo cell

electrofusion, *in vitro* fertilization and cloning of non-human organisms, which are very

complex and controversial aspects of science and medicine to date.

7) Level of skill in the art. The level of skill in the art is low because the

Applicants have not reduced that claimed method to practice.

Given the above analysis of the factors which the Courts have determined are

critical in ascertaining whether a claimed invention is enabled, it must be considered

that the skilled artisan would have had to have practiced undue and excessive

experimentation in order to practice the claimed invention.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form

the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the

applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claim 1 and 17 are rejected under 35 U.S.C. 102(b) as being anticipated by

Magae et al (Appl. Micor. Biotechol., 1986, Vol. 24, 509-511). This is a NEW

REJECTION.

Magae et al teach a method to fuse two giant plant protoplasts by using two

glass electrodes prepared from glass capillaries and attached to a micromanipulator.

Two protoplasts were brought into contact with each other and a single electrical pulse

was applied to the protoplasts to fuse the membranes (see page 509, right column, 3rd

full paragraph) which reads on a method comprising bringing into contact two fusion

partners having cell-like membranes and providing an electric field using at least one

microelectrode which is of a strength to obtain fusion and highly focused on the fusion

partners. The electrodes are positioned by means of a micropositioner and at least one

microelectrode is hollow and sufficiently small to permit the selective fusion of two

fusion partners. Magae et al teach that the protoplast must be prepared in cellulose so

that the protoplasts were large enough for the electrode and were suitable for electrical

fusion in 0.6 M mannitol containing $CaCl_2$ (see page 510, left column, 2nd paragraph,

for example) which reads on the electrode being sufficiently small to permit selective

fusion of the protoplasts and providing the fusion partners in a buffer prior to fusion.

Claims 1- 2, 8-12, 15-19 and 26-29 are rejected under 35 U.S.C. 102(e) as being

anticipated by (Pui et al) U.S. Patent No. 6,093,557, filed 6/5/1998. This is a NEW

REJECTION.

Pui et al teach a method and apparatus for fusion of cells with vesicles or

liposomes that comprises a capillary electrode through which the liposomes are

dispersed in a spray on to target cells (see column 11, lines 6-15, column 14, lines 52-

64, for example). An electrical charge is created between a high voltage liposome-

dispensing capillary tube electrode and a second electrode so that the liposomes are

fused with the target cells (see column 15, lines 14-25, for example). The instant

specification discloses that, in the case of using a single electrode, the electrode is

preferably an anode and works against a grounded cell preparation (see specification

page 9, lines 1-4). Pui et al teach that a high positive voltage can be applied to the first

electrode and the second electrode is grounded, and also teach that the second

electrode can be a grounded ring electrode and a ground target surface holding the

cells (see column 7, lines 5-9 and column 9, lines 46-55, in particular), which reads on

the method wherein only one microelectrode is used to provide the electrical field. Pui

et al teach that the electric field provided between the capillary tube electrode and the

grounded electrode provides for the dispensing of the spray (see column 9, lines 35-45

and column 10, line 40, for example) which reads on a method wherein a small, hollow

electrolyte-filled microelectrode is used to deliver fusion partners by electrophoresis. Pui

et al teach that the spray can be confined to one or more target cells (see column 3,

lines 14-20, column 5, lines 33-35, column 6, lines 26-31, column 6, lines 46-65, in

particular), which reads on bringing into contact at least two fusion partners having cell-like membranes and providing an electric field using at least one microelectrode which is of a strength sufficient to obtain fusion and highly focused on the fusion partners wherein at least one microelectrode is hollow (i.e. capillary tube) and sufficiently small to permit selective fusion of two fusion partners. The spray of liposomes also reads on the method wherein one or more of the fusion partners is constituted by a multiple of a structure such as liposome or vesicle. Pui et al teach that the spray dispenser is movable along the x, y, z axes (see column 17, lines 2-5, in particular) which reads on positioning at least one microelectrode by use of a micropositioner and/or stereotactic device. Pui et al disclose that the outer diameter of the capillary tube has a preferred range of 6 μm to about 2.5 mm, or 8 μm to about 2.5 mm (see column 12, lines 55-60, for example), which reads on an electrode with an outer diameter of 1-100 μm. Pui et al teach that the target cells can comprise cells, eggs or plant protoplasts, may be part of a tissue, or a multilayer of cells (column 13, lines 5-24, and column 17, lines 25-30, for example) which reads on the method wherein at least one of the fusion partners is a cell and the other fusion partners are liposomes, a synthetic vesicle, an egg cell, a plant protoplast or is part of a cellular network. Pui et al teach that the liposome or vesicle particles are in a suspension liquid such as a buffer or electrolyte solution (see column 10, lines 37-41, for example), which reads on the claimed method wherein the fusion partners are provided in a buffer prior to fusion. Pui et al teach that the target cells can be a monolayer of cells as well as being affixed to a surface (see column 13, lines 20-25 and column 22, lines 62-64, for example) which reads on the claimed method wherein

at least one of the fusion partners has been immobilized prior to step A. Pui et al teach that liposomes can fuse to the cell membrane (see column 14, lines 52-65, for example), which reads on manipulation of the composition of the cellular membrane since the lipids of the liposome will now be present in the cellular membrane. Pui et al disclose that controlled flow and a known concentration of biological material, the amount of biological material in the spray can be controlled and is reproducible (see column 14, lines 33-40, for example), which reads on the delivery of a well-defined volume of a substance to a cell. Pui et al teach that DNA, RNA, small molecules, bioactive substances and drugs can be delivered in the carrier particles or liposomes (see column 11, lines 6-14, in particular), which reads on a method of delivery of a pharmaceutically active substance to a cell. Pui et al teach that the delivery method can be used to deliver substances to tumor tissue for gene therapy (see column 17, lines 29-33, for example), which reads on the claimed method for the treatment of a tumor.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Laura McGillem whose telephone number is (571) 272-8783. The examiner can normally be reached on M-F 8:00-5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Irem Yucel can be reached on (571) 272-0781. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the

Patent Application Information Retrieval (PAIR) system. Status information for

published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only.

For more information about the PAIR system, see http://pair-direct.uspto.gov. Should

you have questions on access to the Private PAIR system, contact the Electronic

Business Center (EBC) at 866-217-9197 (toll-free).

Laura McGillem, PhD
2/27/2006

DAVID GUZO
PRIMARY EXAMINER

Interview Summary	Application No.	Applicant(s)
	10/031,410	ERIKSSON ET AL.
	Examiner	Art Unit
	Laura McGillem	1636

All participants (applicant, applicant's representative, PTO personnel):

(1) _Laura McGillem_.   (3)_David Guzo_.

(2) _Stephana Patton_.   (4)_____.

Date of Interview: _2/7/2006_.

Type: a)☒ Telephonic   b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant   2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes   e)☐ No.
If Yes, brief description: _____.

Claim(s) discussed: _1,30 and 34_.

Identification of prior art discussed: _____.
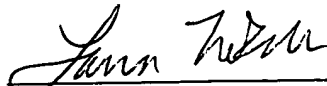
Agreement with respect to the claims f)☒ was reached.   g)☐ was not reached.   h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: _See Continuation Sheet_.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

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1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

 Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: This interview regarded amendments to the claims that had been submitted in the After final amendment, filed 1/6/2006 and subsequently not entered because the amendments broadened the scope of the claims. Attorney Patton inquired whether restoration of the phrase "at least two fusion partners having cell-like membranes" would be more acceptable in order to overcome the prior art rejections. Examiners suggested that the amendment to claim 1 to add the limitation "wherein at least one microelectrode is hollow, and sufficiently small to permit the slective fusion of two fusion partners" might be more effective to overcome the prior art rejections, subject to further search and examination. Examiner McGillem mentioned that rejections of claims 30 and 34 under 35 USC 112, 2nd paragraph has not been sufficiently overcome. Attorney Patton agreed to cancel claims 30 and 34. Attorney Patton will submit a Supplemental amendment.

	Application No.	Applicant(s)
Interview Summary	10/031,410	ERIKSSON ET AL.
	Examiner	Art Unit	
	Laura McGillem	1636	

All participants (applicant, applicant's representative, PTO personnel):

(1) _Laura McGillem_.   (3)_____.

(2) _Stephana Patton_.   (4)_____.

Date of Interview: _24 February 2006_.

Type: a)☒ Telephonic   b)☐ Video Conference
      c)☐ Personal [copy given to: 1)☐ applicant   2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes   e)☒ No.
If Yes, brief description: _____.

Claim(s) discussed: _15,16,23 and 24_.

Identification of prior art discussed: _____.

Agreement with respect to the claims f)☐ was reached.   g)☒ was not reached.   h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: _See Continuation Sheet_.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

_____
(Examiner's signature, if required

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Attorney Patton was contacted regarding claims 23-24 which are drawn to methods of in vitro fertilization and non-human cloning. Examiner McGillem pointed out that the specification was not enabled for methods of in vitro fertilization and cloning. Attorney Patton agreed to cancel claims 23-24. Examiner McGillem suggested that in claims 15-16 the recitation of fusion partners consisting of "an egg cell, an enucleated egg cell and a sperm cell at any development stage" should be removed from the claims because they relate to methods of in vitro fertilization and cloning. Attorney Patton submitted that the cells could be used for experimental methods other than cloning or fertilization, and said that the inventors would be contacted for their opinion

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
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2

Notice of References Cited	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10/031,410	ERIKSSON ET AL.

	Examiner	Art Unit	
	Laura McGillem	1636	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,093,557	07-2000	Pui et al.	435/173.1
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
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	L	US-			
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FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Magae Y, Kashiwagi Y, Senda M, Sasaki T. 1986. Electrofusion of giant protoplasts of Pleurotus cornicopiae. Appl. Micorbiol Biotechnol. 24:509-511
	V	Sakai RR, Tamashiro KL, Yamazaki Y, Yanagimachi R. 2005. Cloning and assisted reproductive techniques: influence on early development and adult phenotype. Birth Defects Res C Embryo Today. Jun;75(2):151-62.
	W	Mekid H, Mir LM. 2000. In vivo cell electrofusion. Biochim Biophys Acta. Dec 15;1524(2-3):118-30.
	X	Orentas RJ, Schauer D, Bin Q, Johnson BD. 2001. Electrofusion of a weakly immunogenic neuroblastoma with dendritic cells produces a tumor vaccine.Cell Immunol. Oct 10;213(1):4-13.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

		Application/Control No.	Applicant(s)/Patent Under Reexamination
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		Examiner	Art Unit	Page 2 of 2
		Laura McGillem	1636	

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	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Niemann H, Rath D. 2001. Progress in reproductive biotechnology in swine.Theriogenology. 2001 Nov 1;56(8) Abstract.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Short contribution

Electrofusion of giant protoplasts of *Pleurotus cornucopiae*

Yumi Magae[1], Yutaka Kashiwagi[1], Mitsugi Senda[2], and Takashi Sasaki[1]

[1] National Food Research Institute, P.O. Box 11, Tsukuba Science City, Ibaraki 305, Japan
[2] Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Summary. Giant protoplasts of *Pleurotus cornucopiae* were fused, using the glass microelectrode electrofusion technique; the percentage fusion achieved was 70%. To induce fusion, Ca^{2+} was necessary, a 10 mM concentration giving the best result. Polyethylene glycol 4000 (PEG) promoted fusion but also increased the adhesion of protoplasts, which caused them to be irreversibly attached to the electrodes. Fusion was always completed within 1 min after a single electrical pulse had been applied. The fused protoplast was isolated with a glass micropipette and was found to regenerate into a colony.

Introduction

Although protoplast fusion techniques offer the potential for the production of novel hybrids that cannot be obtained by sexual means, much time has to be spent in developing a selection system of fusion hybrids. This technique has therefore mostly been successful only with those organisms for which appropriate mutants are readily available. In this respect, the electrofusion technique using glass capillary microelectrodes reported by Senda (1979) has some advantages over other fusion methods if the cells are large enough for the introduction of microelectrodes; fusion can be directly observed under the microscope and the fusant can be directly transferred to a nutrient medium without any need of selection media.

Enlargement of protoplasts of *Pleurotus cornucopiae*, as reported in previous work (Wakabayashi et al. 1985), enabled the introduction of this method to filamentous fungi for the first time. In

Offprint requests to: T. Sasaki

this paper the first results of electrofusion of *P. cornucopiae* protoplasts are described.

Materials and methods

Organism. Pleurotus cornucopiae IFO9614 used in this study was obtained from Institute for Fermentation, Osaka, Japan.

Formation of giant protoplasts. The preparation of giant protoplasts of *Pleurotus cornucopiae* has been described in detail (Wakabayashi et al. 1985). Briefly, purified protoplasts were incubated in 5% cellulase ONOZUKA RS in 0.6 M mannitol—0.05 M phosphate buffer (pH 5.6) at 24°C for several days. They were centrifuged and washed with 0.6 M mannitol containing $CaCl_2$ before subjected to electrofusion.

Electrofusion. Electrofusion was carried out according to the method of Senda (1979). The glass electrode was prepared with a glass capillary (1.0×90 mm Narishige, No. G-1) drawn by a micropipette puller (Narishige No. PW-6) and filled with 3 M KCl. A drop of protoplast solution was pipetted onto a cover glass (22×22 mm) and it was set on the stage of an inverted microscope (Olympus). A single electrical pulse was applied discharging a capacitor through a resistor in parallel to two protoplasts in contact between the microelectrodes attached to micromanipulator (Narishige No. MN-100, MO-104). Fusion frequency was estimated as the number of fused protoplasts that had achieved the final stage; that is, a completely spherical form, among 10 to 20 pairs of protoplasts.

Isolation of fusion products. When protoplasts were fused to spherical form, they were picked up by micromanipulation with a glass micropipette which was filled to the tip with sterilized liquid paraffin and connected to a microinjector (Narishige No. IM-4). The tip of the micropipette was cut to have about 50 µm outer diameter opening. The fusant was picked up by weak negative pressure and then released onto 0.5% agar regeneration medium (1.5% yeast extract, 1.5% peptone, 0.6 M sucrose) in a plastic Petri dish (Corning, 35 mm).

Results and discussion

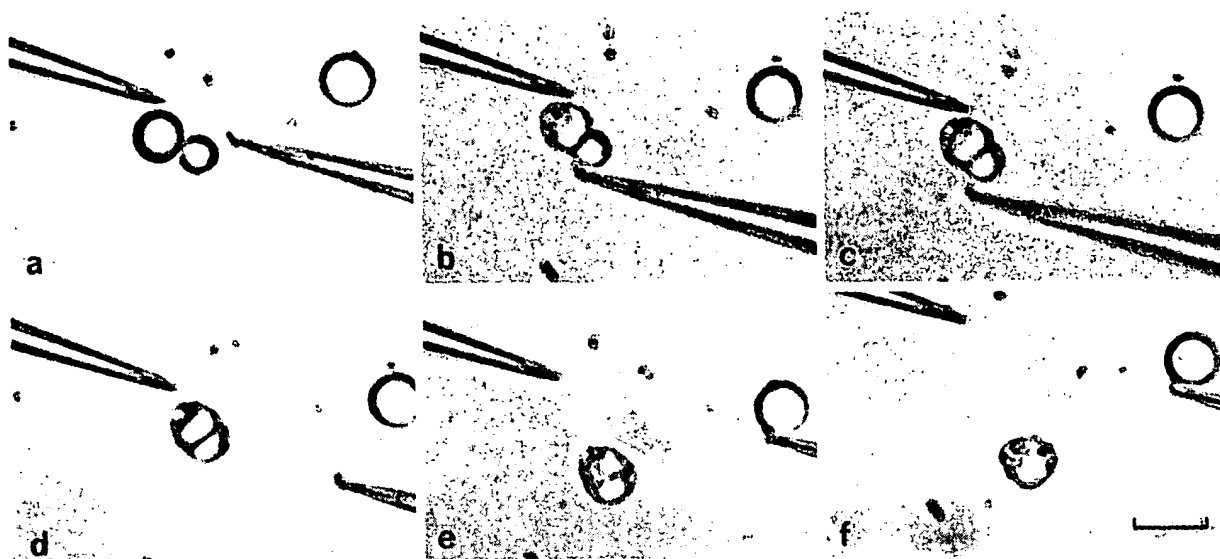Electrical stimulation alone has never sufficed to induce fusion of protoplast membrane: the pres-

Fig. 1. Two giant protoplasts of *Pleurotus cornucopiae* were induced to fuse in 0.6 M mannitol—10 mM $CaCl_2$ through an electrical stimulation. **a** Protoplasts in contact before the pulse application. **b, c, d** Proceeding to face adherent stage after a pulse was applied. **e, f** Forming fused spherical shape

ence of Ca^{2+} was found to be essential when a pulse was applied to protoplasts in 0.6 M mannitol. When fusion frequency of protoplasts in 0.6 M mannitol containing 0 to 15.0 mM $CaCl_2$ was determined, a $CaCl_2$ concentration of more than 5 mM was found to be necessary to achieve the final spherical shape. At concentrations greater than 12.5 mM, however, change in the appearance of protoplast surface occurred as if the rigidity of the membrane increased, which possibly was the reason for the decrease of fusion frequency. A concentration of 5 mM $CaCl_2$ gave the highest frequency of 70%, but at this concentration protoplasts showed a tendency to attach to the electrodes, resulting in deformation of the fused cells. Cells were more stabilized in 10.0 mM. Therefore in further experiments 0.6 M mannitol with 10 mM $CaCl_2$ was used.

After 2 days' incubation in 5% cellulase ONO-ZUKA RS, protoplasts were large enough (average diameter 17 μm) for the electrodes and were most suitable for electrical fusion. Although longer incubation in the cellulase produced larger protoplasts, they failed to proceed further than the face adherent stage after a pulse was applied. Fusion occured when a current pulse at the voltage between 250 and 400 V lasting 5 msec was applied to contacting protoplasts in 0.6 M mannitol—10 mM $CaCl_2$ (Fig. 1).

Polyethylene glycol (PEG) at a concentration of 5%—15% apparently increased the number of protoplasts in contact; the fusion process was

promoted with 10% PEG. Adherence of cell surface also increased, however, so that the fusants remained attached to the microelectrodes or the cover glass. In conclusion, there were no practical merits in adding PEG to the solution.

With plant protoplasts, the fusion process proceeds rather gradually, taking 10 to 30 min (Senda et al. 1979, 1982). It was much faster, presumably due to its smaller size, with *P. cornucopiae* protoplasts. The average times taken for seven successive trials ranged from 1.7 to 0.35 s to attain the face-adherent state and between 29.2 and 16.3 s to the final fused-sphere stage. As it takes less than 1 min for each fusion process, the rate limiting step of whole experiment is the picking up of each cell and the fusant. This means a device is required which will allow precise control of the micromanipulation of the cell.

Protoplasts fused to spherical form and picked up by micromanipulation with a glass micropipette were put on the medium in a small droplet of liquid paraffin. When observed under the light microscope after 4 to 6 days' incubation at 24°C, they were found to grow into small colonies. The appearance of growing hyphae was not different from the normal *P. cornucopiae* so far. The ability of fusants to regenerate has been ascertained for several times and was found to be highly reproducible.

If the regeneration rate of the organism is normal, electrofusion with two microelectrodes appears to offer marked practical advantages over

other methods because only one to one fusion of the two cell types can occur. Protoplast fusion experiments using PEG always produce a mixture of self to self hybrids and the true fused cells. This gives rise to a need for a nutrient or drug resistant marker as well as special selective media. It has also been the case with other fusion techniques, such as the electrofusion method using an alternating electric field (Halfmann et al. 1983; Watts et al. 1985) first reported by Zimmermann (1981), because aggregates of several protoplasts derived from the same origin appear during the fusion procedure. With the present microelectrode method, however, no marker is needed, for protoplasts can be picked one by one from different cultures just before the electrical stimulation.

This method can, however, only be used for protoplasts large enough for the introduction of microelectrodes. If the size of protoplast cells could be increased as with *P. cornucopiae*, it would be possible to use this microelectrode electrofusion technique even with microorganisms.

Since self to self protoplast fusion of *P. cornucopiae* and also *P. ostreatus* (data not shown) has been successful in our laboratory, study of interspecific fusion is now in progress.

References

Halfmann HJ, Emis CC, Zimmermann U (1983) Electro-fusion of haploid *Saccharomyces* yeast cells of identical mating type. Arch Microbiol 134:1—4

Senda M, Takeda J, Abe S, Nakamura T (1979) Induction of cell fusion of plant protoplasts by electrical stimulation. Plant Cell Physiol 20:1441—1443

Senda M, Morikawa H, Takeda J (1982) Electrical Induction of cell fusion of plant protoplasts. Proc. 5th Intl Cong Plant Tissue Cell Culture 615—616

Wakabayashi S, Magae Y, Kashiwagi Y, Sasaki T (1985) Formation of giant protoplasts of *Pleurotus cornucopiae* by the cell wall lytic enzyme. Appl Microbiol Biotechnol 21:328—330

Watts JW, Doonan JH, Cove DJ, King JM (1985) Production of somatic hybrids of moss by electrofusion. Mol Gen Genet 199:349—351

Zimmermann U, Scheurich P (1981) High frequency fusion of plant protoplasts by electric fields. Planta 151:26—32

10/148,457

REVIEW

Cloning and Assisted Reproductive Techniques: Influence on Early Development and Adult Phenotype

Randall R. Sakai,* Kellie L. K. Tamashiro, Yukiko Yamazaki, and Ryuzo Yanagimachi

Over the past 40 years, our increased understanding and development of cell and molecular biology has allowed even greater advances in reproductive biology. This is most evident by the development of various aspects of assisted reproductive techniques (ART), generation of transgenic animals, and most recently generation of mammals through somatic cell cloning. To date, cloning from adult somatic cells has been successful in at least 10 mammalian species. Although generating viable cloned mammals from adult cells is technically feasible and the list of successes will only continue to grow with time, prenatal and perinatal mortality is high and live cloned offspring have not been without health problems. The success of many of the proposed applications of the cloning technique obviously depends upon the health and survival of founder animals generated by nuclear transfer. This article summarizes the health consequences of cloning in mice, and discusses possible mechanisms through which these conditions may arise. In addition, we discuss the effects of ART in animal models and in humans. ART also involves some of the same procedures used in cloning, and there are reports that offspring generated by ART sometimes display aberrant phenotypes as well. It is important to point out that although these techniques do sometimes produce abnormalities, the majority of offspring are born apparently normal and survive to adulthood. Additionally, we must emphasize that the effects of ART and cloning observed in animal models do not necessarily indicate that they will occur in humans. In this article, we review studies examining the phenotype of animals generated by cloning and various ART, and discuss clinical implications of these findings. **Birth Defects Research (Part C) 75:151–162, 2005.**
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INTRODUCTION

The "Barker hypothesis" proposes that the embryonic and fetal environments have strong influences on long-term health consequences (Hales and Barker, 1992). Developmental biological studies indicate that various physiological systems and organs have critical windows of development and can be irreversibly influenced by environmental conditions during those periods. Indeed, evidence is accumulating that supports the notion that an altered early developmental environment can predispose offspring to disorders such as diabetes mellitus, obesity, cardiovascular disease, stroke, depression, and schizophrenia (Young, 2001).

Analogously, the same principle also applies to offspring generated by assisted reproductive techniques (ART), including somatic cell cloning, since these procedures necessitate exposing developing fetuses to a different environment. It is clear from current literature that preimplantation embryos are sensitive to environmental conditions and are predisposed to developing altered phenotypes during the postnatal period and as adults. In vitro studies have established that embryo exposure to suboptimal culture media, for example, results in decreased fetal growth relative to in vivo–derived counterparts. Further studies have identified specific components of culture media that may be responsible for adverse effects on embryos. This is just one example of how an altered or artificial environment can have long-term phenotypic consequences. In addition, mechanical handling and injection of sperm or somatic cell nuclei further challenge normal development of the early embryo and fetus.

Several mammalian species have been cloned by somatic cell nuclear transfer, and the list of proposed

clinical and commercial applications continues to grow. Additionally, and perhaps more importantly, the use of ART in humans has continued to increase steadily (CDC, 2002). However, the safety of cloning and ART and their short- and long-term effects on offspring remain largely unknown. In this article, we review studies examining the phenotype of animals generated by cloning and various ART, and we discuss the clinical implications of these findings.

CLONING

Cloning can occur naturally in plants and lower invertebrates, and has been used for centuries to produce genetically identical offspring. More recently, cloning has been applied to several mammalian species as well (Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998; Polejaeva et al., 2000; Chesne et al., 2002; Keefer et al., 2002; Shin et al., 2002; Galli et al., 2003; Woods et al., 2003; Zhou et al., 2003). Potential applications of the technology include production of genetically engineered cattle and goats, for example, to mass-produce pharmaceuticals in milk (Wilmut and Paterson, 2003). Because traditional transgenic methods are ineffective in livestock, cloning provides an alternative means through which cattle and goats with desired phenotypes can be generated (Wilmut and Paterson, 2003). Pigs are valuable sources of replacement tissues and organs, and have been engineered to provide compatible tissues for use in humans (Lai et al., 2002; Wilmut and Paterson, 2003). Another application that has been proposed is propagation of endangered species (Holt et al., 2004) and beloved family pets such as cats and dogs (Westhusin et al., 2001; Shin et al., 2002; Westhusin et al., 2003).

Cloning by nuclear transfer of cell nuclei can be considered the most extreme version of assisted reproduction. The successful application of cloning technology for human reproduction purposes has been claimed by some (Vogel, 2001); however, there is no evidence that

it has indeed occurred, or is even possible (Schatten et al., 2003). Nevertheless, the technology will continue to move forward, and the ability to generate a human clone may one day be possible.

The success of many of the proposed applications of the cloning technique obviously depends upon the health and survival of founder animals generated by nuclear transfer. Systematic studies of cloned offspring are imperative in order to establish the method's safety and usefulness. This is not a simple task given the low rate of cloning (the proportion of live offspring resulting from all nuclear transfer eggs), which currently stands at 2–3% at best, regardless of species or nuclear transfer technique. Obtaining sufficient numbers of animals to conduct comprehensive studies is therefore a challenge for most experimental species. However, despite this technical obstacle, several groups have conducted follow-up studies of limited numbers of cloned animals, and an important preliminary conclusion is that clones are not always phenotypically identical to their somatic cell donors and that they often develop adverse health conditions after birth (Renard et al., 1999; Tamashiro et al., 2000, Tamashiro et al., 2002; Tamashiro et al., 2003; Lanza et al., 2001; Edwards et al., 2002; Govoni et al., 2002; Lee et al., 2003). While many studies focused on behavioral and physiological measures to assess the consequences of cloning, others have examined telomere length (Shiels et al., 1999; Lanza et al., 2000; Tian et al., 2000; Wakayama et al., 2000; Betts et al., 2001), genomic imprinting and DNA methylation (Cho et al., 2001; Inoue et al., 2002), and mitochondrial DNA genotype (Evans et al., 1999). The findings of all of these reports are consistent with the hypothesis that aberrations occur in cloned animals, aberrations that could have detrimental consequences.

Although most studies of cloned animals suggest that, in many cases, clones are not normal, there has been little consistency or con-

sensus as to the exact nature of the problem. The reason for this is that thorough studies of cloned offspring are often limited by the ability to generate a sufficient number of age-matched cloned animals and adequate control groups. Adding to the complexity of the problem, while some clones may exhibit changes and phenotypic differences immediately after nuclear transfer and at birth, there is sufficient evidence that altered phenotypes may become apparent only during adulthood or may remain undetected throughout the animal's lifetime (Chavatte-Palmer et al., 2002; Cibelli et al., 2002; Wilmut, 2002). An important point is that the cloning technique is still unpredictable, highlighting the need for comprehensive and systematic longitudinal studies of cloned animals.

As with any scientific endeavor, appropriate controls are of paramount importance. What are appropriate controls for studies of cloned animals? Age- and strain-matched control groups have been included in many studies; however, cloning by somatic cell nuclear transfer (SCNT) bypasses virtually all of the biological processes that occur with natural, sexual reproduction. It is therefore important to ask whether an animal produced by natural mating is a sufficient control. Recent studies have included "manipulated" controls or some other variation to attempt to control for some of the differences between the procedures and to dissect out the effects of each technique. It is important to recognize the limitations of some of the control groups that are used in studies of cloned animals and to consider this as interpretations are made. As an example, in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), or in vitro culture of in vivo produced embryos is often used to generate control groups. However, animals produced by these methods also exhibit significant differences when compared to animals derived from natural mating, suggesting that these procedures alone have significant effects on offspring (Tama-
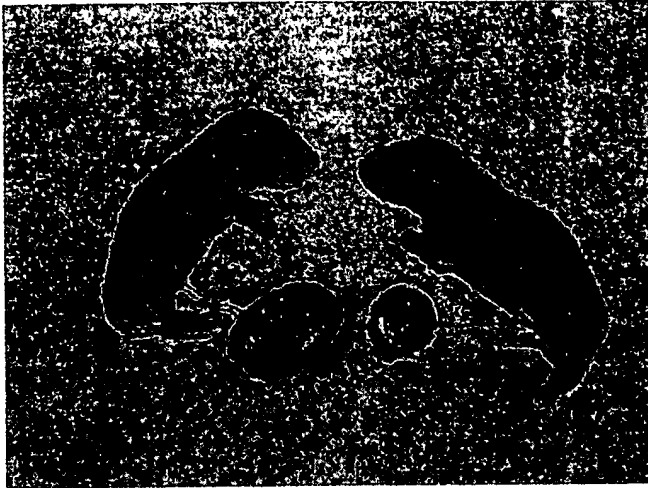
Figure 1. Placenta of cloned and control mice at birth. Typical term placentae in cloned mice (left) are two to three times heavier than those of control mice (right). Adapted from Tamashiro et al. (2005).

shiro et al., 2000, Tamashiro et al., 2002; Ogonuki et al., 2002; Ecker et al., 2004). Therefore, comparisons of clones to IVF or ICSI groups may yield small differences or no significant difference at all by masking effects that would have been revealed had natural, in vivo-produced control groups been included for comparison. It is critical that the appropriate control groups are included in order to differentiate clone-specific phenotypes from those resulting from technical aspects of the technique.

In this report, we review studies that have been conducted to determine the short- and long-term consequences of somatic cell cloning. The health of adult clones has been most extensively studied in mice. Although longitudinal studies of cloned livestock, primarily cattle and pigs, are now being conducted, the long-term consequences will not be known for years due to the long lifespan of these species. We also discuss the implications of manipulations and procedures involved in cloning as they relate to ART and its impact on offspring, both in animal models and in humans.

Embryonic Development and the Perinatal Phenotype

Pre- and perinatal death rates are significantly higher in clones com-

pared to controls regardless of species (Wells et al., 2004). In the mouse, investigators have observed high embryo implantation rates (57–71%), but low fetal (5–16%) and very low full-term (2–3% or less) development rates following nuclear transfer using adult somatic cells (Wakayama et al., 1998; Yanagimachi, 2002). Cloned cattle also suffer from high mortality during prenatal and perinatal periods, limiting the overall efficiency of cloning in this species to 5–6% at best (Chavatte-Palmer et al., 2004; Wells et al., 2004). The reason(s) for the low efficiency of somatic cell cloning are currently unclear, and is the subject of extensive research, particularly in the field of genomic reprogramming.

Perinatal and postnatal complications in mice primarily consist of developmental deficiencies and respiratory distress (Wakayama and Yanagimachi, 1999, Wakayama and Yanagimachi, 2001; Eggan et al., 2001; Ogura et al., 2002). In cattle, significant postnatal losses within the first six months affect about 30% of clones that develop to term. The causes of death include pulmonary difficulties and respiratory distress at birth, abnormal kidney development, and liver steatosis (Chavatte-Palmer et al., 2004). Other groups report higher incidence of hydrallantois, difficulty of parturition, and neonatal death

among clones than in in vitro-produced controls (Matsuzaki and Shiga, 2002). Matsuzaki and Shiga (2002) examined plasma concentrations of cortisol, adrenocorticotropic hormone (ACTH), and components of the insulin-like growth factor (IGF) system in order to assess the possible link between endocrine status and perinatal anomalies related to cloning. Their data indicate that in clones there is a significantly reduced rise in plasma cortisol that is insufficient to switch the IGF system to "adult mode" during late gestation, resulting in difficulties during parturition. Overall, their data suggest that inappropriate or insufficient changes during development may be partially responsible for fetal overgrowth and perinatal complications associated with the cloning process.

Placentomegaly is one striking and consistent characteristic of cloned mice (Wakayama et al., 1999, Wakayama et al., 2000; Tanaka et al., 2001; Ogura et al., 2002; Singh et al., 2004) (Fig. 1). In cloned mice, placentae are enlarged by approximately two- to three-fold over those of controls, irrespective of gender of clone, nuclear donor source, or nuclear transfer protocol (Wakayama et al., 1999; Ogura et al., 2000). Abnormal placentae have also been noted in cattle (Cibelli et al., 1998), and are thus not unique to cloned mice.

Placental abnormalities in humans have been associated with adverse effects on growth and development of the fetus. Thus, identifying the pathways and genes involved in normal placentation is of great interest. The possible mechanisms responsible for larger placental size have been more thoroughly studied in cloned mice, and hypertrophy of the basal layer, spongiotrophoblasts, giant trophoblasts, and glycogen cells have all been associated with this phenotype (Tanaka et al., 2001). In addition, placental zonation is disrupted, and is characterized by interdigitation of the labyrinthine-basal layer boundary and disorganization of the labyrinthine layer (Tanaka et al., 2001). Similar placental abnormalities have been observed in
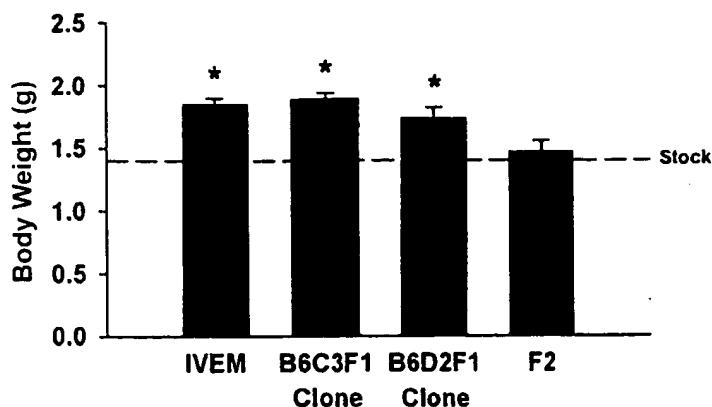
Figure 2. Birth weights of cloned, control, and F2 mice. *$P < 0.05$ versus stock and F2. Adapted from Tamashiro et al. (2002).

mice produced by other in vitro micromanipulation techniques, such as ICSI, round spermatid injection (ROSI), aggregation chimera, and pronuclear exchange (Ogura et al., 2002), suggesting that some aspect of the in vitro environment or embryo manipulation is responsible for the placental phenotype, and that it is not specific to cloning. However, only cloned mice exhibited basal layer expansion associated with marked proliferation of glycogen cells, suggesting that nuclear transfer is responsible for these specific characteristics (Ogura et al., 2002). The mechanisms for placental hypertrophy in cloned mice remain unclear.

Increased birth weights have been reported in cloned mice, sheep, and cattle (Chavatte-Palmer et al., 2002; Pace et al., 2002; Tamashiro et al., 2002) (Fig. 2). This so-called "large offspring syndrome" (LOS) has also been described in detail in animals that were exposed to culture medium as embryos (Young et al., 1998, Young et al., 2001). Some investigators report clone birth weights that did not differ from those of "controls"; however, the control groups were produced by IVF or ROSI, both of which involve periods of in vitro exposure to culture medium (Ogura et al., 2002), and thus both clones and IVF and ROSI controls may have been heavier than in vivo–derived controls.

Postnatal Development and Behavior

Despite the high mortality rate prior to and at birth, there are apparently healthy cloned animals that survive to adulthood. Unfortunately, few systematic studies of the behavior of cloned animals have been conducted thus far. Behavioral studies in species such as cattle and pigs are inherently difficult; however, cloned mice are an ideal model to use for this purpose. Our group has conducted some of the first systematic behavioral studies with reasonable numbers of subjects in cloned animals. These studies included two control groups. One control group (designated in vitro embryo manipulated [IVEM]) was generated to account for many of the manipulations and procedures unique to the cloning process such as in vitro culture, embryo transfer, Cesarean section delivery, and cross-fostering. The second control group consisted of age- and strain-matched mice derived by natural mating (designated "STOCK") (Tamashiro et al., 2000).

We examined the postnatal development of cloned mice and IVEM controls according to established developmental criteria, and found no differences between cloned and IVEM mice with the exception of negative geotaxis, ear twitching, and eye opening (Tamashiro et al., 2000). Both groups still fell well within the normal range reported

for mice, and our subsequent behavioral tests did not reveal any deficits or long-term consequences in the clones that may be related to delay in development of these milestones. The milestones and behaviors included in our examination are normally expressed at different time points during the first 21 days of life, and thus provide a measure of development that spans the entire postnatal pre-weaning period in mice (Fox, 1965).

Home cage activity does not differ between clones and IVEM control mice at any of the time points examined, suggesting that cloned mice have normal diurnal activity patterns (Tamashiro et al., 2000). Additionally, we assessed motor skills and abilities of the clones and did not find any deficits in motor coordination, muscle strength, or balance. In sum, cloned mice appear to develop reflexes and other behaviors at the same ages as normal mice, and to have normal motor control and coordination (Tamashiro et al., 2000).

To evaluate learning and memory of cloned mice, we used a well-characterized and widely used task, the Morris water maze (Morris, 1981). Both clones and IVEM controls successfully completed the task, finding the submerged platform with a shorter latency over consecutive days of testing. There were no differences in performance between the groups, suggesting that both groups were able to use information obtained from previous trials to find the platform faster on subsequent days. These results also indicated that both groups were employing a spatial learning strategy, as indicated by the increased amount of time spent in the quadrant where they had been trained to find the platform. Furthermore, when the position of the submerged platform was changed, the cloned mice navigated to the new platform position and found it with a shorter latency than during the initial acquisition trials. Together, these results suggest that cloned mice are capable of completing a spatial learning task and do not have deficits in learning and memory, at least through six
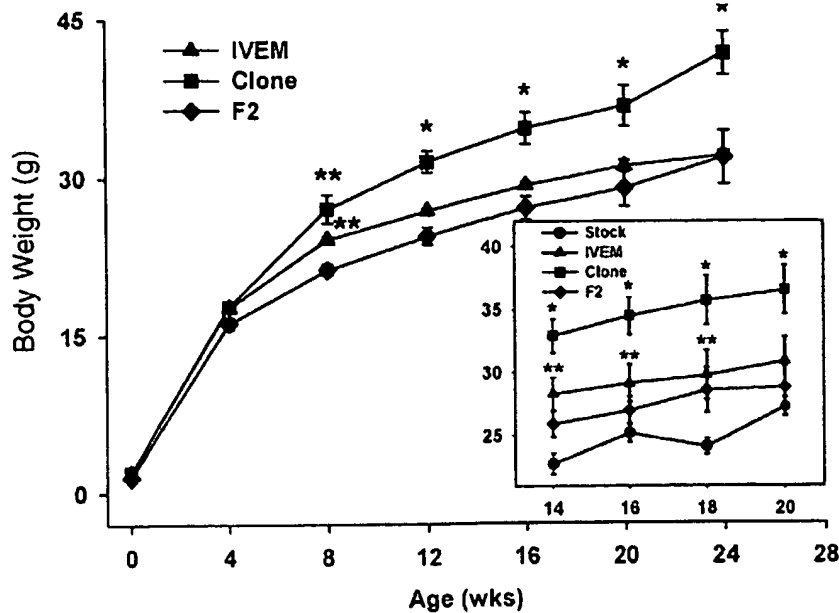
Savage et al. (2003) reported that adolescent cloned calves do not have behavioral indications of premature aging such as decreased activity levels, but tended to "play" less than age-matched controls produced by artificial insemination (AI). Cloned cattle also had higher levels of curiosity and more self-grooming and aggressive behaviors than controls. It is important to note that the sample size in these studies was small (n = 4), and a larger cohort of cloned cattle needs to be examined. Furthermore, whether these differences in behavior have significant effects beyond adolescence remains to be determined.

Physiology

Although cloned mice appear to be indistinguishable from controls through six months of age, cloned mice weighed significantly more than age-matched controls beginning at approximately 8–10 weeks of age (Fig. 3) (Tamashiro et al., 2000). Likewise, other investigators have found that female cloned mice of the B6D2F1 strain also have higher body weights compared to control animals (T. Wakayama, unpublished observations). Ogura et al. (2002) reported increased body weight in B6 X 129 male mice cloned from Sertoli cells, suggesting that higher body weight in clones is not a strain-specific occurrence. Likewise, B6D2F1 male mice cloned from fetal neurons have elevated body weight compared to age-matched STOCK control mice (Tamashiro et al., 2003, Tamashiro et al., 2005). Body composition analyses indicated that increased body weight is associated with increased adipose tissue, and consistent with these data, clones were also hyperinsulinemic and hyperleptinemic. As adults, cloned mice were not hyperphagic and responded normally to an acute hypocaloric challenge. Interestingly, cloned mice have hypersensitive leptin and melanocortin signaling systems, suggesting that defects within this system cannot account for obesity in cloned mice (Tamashiro et al., 2002). The mecha-
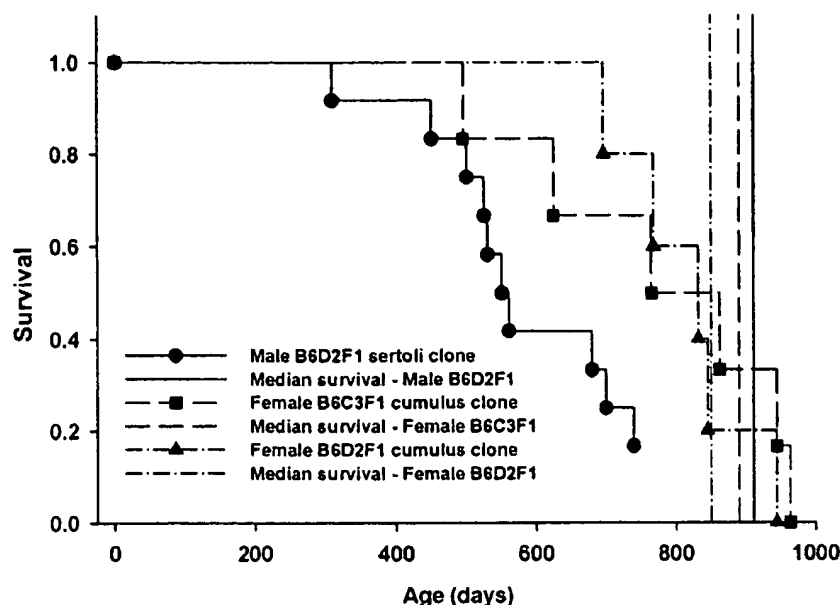
Figure 3. A: Body weight of cloned, IVEM, and F2 offspring mice. *P < 0.05 versus IVEM and F2; **P < 0.05 versus F2. Inset, comparison of body weight of clones, IVEM, and F2 to age-matched background stock mice at 14–20 weeks of age. *P < 0.05 versus IVEM, F2, and stock; **P < 0.05 versus stock. Adapted from Tamashiro et al. (2002). **B:** Photograph of age- and strain-matched cloned and control mice. An adult female B6C3F1 cloned mouse (bottom left) and representatives of control groups: stock (foreground) and IVEM (in vitro embryo manipulated) (top middle).

months of age (Tamashiro et al., 2000).

Overall, these behavioral data suggest that cloned mice are not significantly different from control mice from birth through six months of age. We have also examined multiple generations of cloned mice and have not noted any significant deficiencies in any of the developmental or behavioral measures described above (Wakayama et al., 2000). Further longitudinal studies of behavior over the entire lifespan

of cloned mice have yet to be conducted. It is thus possible that cloned animals may develop behavioral deficits later in life.

Behavioral observations of cloned cattle suggest that they exhibit normal social interactions and behavior, including development of a social dominance hierarchy (Lanza et al., 2001). This report was somewhat subjective, since no behavioral measures were presented and noncloned control groups were not included.

Figure 4. Longevity of female mice cloned from adult cumulus cells. Adapted from Tamashiro et al. (2003).

nism(s) responsible for the development of obesity in cloned mice remains to be determined, and is the subject of active research in our laboratory. Although increased body weight has not been reported in domestic species, at least one study reports that observations made during postmortem examination indicates that cloned fetuses and newborn calves had more adipose tissue surrounding the intra-abdominal organs, as compared with controls. Plasma leptin was also elevated, consistent with increased adipose tissue content (Chavatte-Palmer et al., 2002).

Lanza et al. (2001) studied 24 cloned cattle and reported normal hematology, biochemistry, and lymphocyte populations; however, contemporary control groups were missing from this study. Wells et al. (2004) also examined a set of cloned cattle and their contemporary controls at two years of age. Although this group reported significant postnatal losses during the first six months after birth, they found no significant differences in tests of hematological parameters, or indicators of muscle injury, liver function, serum proteins, renal perfusion, and metabolic status in those clones that survived to two years of age. It is important to note

that this is one of only a few longitudinal studies that are being conducted with contemporary control groups, and that these animals were assessed at one time point early in their lifetime. Likewise, Chavatte-Palmer et al. (2002) found no significant differences in hematologic parameters in clones compared to AI controls. However, they also conducted a battery of assays to assess the endocrine status of cloned cattle during perinatal and postnatal development, and found that plasma T4 and IGF-II levels were decreased in clones while leptin level was elevated (Chavatte-Palmer et al., 2002). Future reports from these groups of animals are required to determine whether aberrations in cloned cattle will be manifest later in life.

Longevity

The use of differentiated adult somatic cells for cloning calls to question the actual age of a cloned animal. Studies have examined telomere lengths as an indirect measure of aging in cloned animals, since progressive shortening of telomeres during DNA replication and cell division has been associated with cellular aging or senescence (Shay and

Wright, 2000). Cloned mice do not have behavioral deficits or signs of premature aging (Tamashiro et al., 2000, Tamashiro et al., 2003; Wakayama et al., 2000). To further investigate aging in cloned mice, Wakayama et al., successfully generated four and six generations of cloned mice in two independent lines, i.e., clones derived from clones (Wakayama et al., 2000). If cloning mice from adult somatic cells results in short telomeres, serial generations of cloned mice should have telomeres that become progressively shorter. Behaviorally, the six generations of cloned mice did not exhibit signs of premature aging. Telomere lengths were also analyzed in this experiment to provide a molecular measure of aging. Consistent with the behavioral results, no telomere shortening was observed. In fact, telomere size increased with each successive generation (Wakayama et al., 2000).

Ogonuki et al. (2002) reported that a majority of their B6D2F1 male clones derived from immature Sertoli cells died at about 500 days, approximately 50% of the normal mouse lifespan. Other reports, however, show that mice cloned from other cell types and on a different background strain have normal lifespans (Ogura et al., 2002; Tamashiro et al., 2003) (Fig. 4). Histopathology of our cumulus cell clones indicated that most of the cloned mice died of conditions associated with normal aging, including leukemia, myocardial degeneration, adenocarcinoma, and lymphoma (Tamashiro et al., 2003). In fact, the first cloned mouse, Cumulina, lived for two years and seven months, a very decent age for a mouse with an average two-year expected lifespan (Aldhous, 2000). It thus appears that the early death in cloned mice observed by Ogonuki et al. (2002) may be strain and/or cell type specific, at least in the mouse. In addition, the high mortality rate in that study was attributed to higher susceptibility to conditions and diseases, mainly pneumonia and hepatic failure, stemming from reduced immunological function. Immunosuppres-

sion has also been reported in cloned cattle (Renard et al., 1999) and goats (Keefer et al., 2001), suggesting that this condition is not unique to mice. Previous studies indicate that telomere shortening may contribute to immunological dysfunctions in mice (Blasco, 2002). Telomere lengths were not measured by Ogonuki et al. (2002); however, given the degree of variability found in telomere lengths among cloned cattle (Lanza et al., 2000; Tian et al., 2000; Betts et al., 2001; Miyashita et al., 2002), which may be dependent upon the donor cell type, it is plausible that the mice in this particular experiment had shortened telomeres leading to premature immune insufficiency.

Cloned sheep were reported to have shorter telomeres suggestive of premature aging (Shiels et al., 1999). Wilmut and colleagues (Wilmut and Paterson, 2003) reported that Dolly, in addition to having shorter telomeres, exhibited characteristics of advanced aging such as early development of arthritis. She was euthanized at six years of age, about one-half the normal lifespan of a sheep, because she was suffering from a virus that caused a tumor in her lung (Giles and Knight, 2003). In contrast, cattle had either age-appropriate (Tian et al., 2000; Kubota et al., 2004) or longer telomeres (Lanza et al., 2000). Current studies in cattle report normal survival of four-year old bovine clones; however, it may be too soon to detect a shorter lifespan in this species since cattle have a potential lifespan of at least 20 years (Lanza et al., 2001). Similarly, cloned pigs have telomere lengths that are comparable to their age-matched controls (Jiang et al., 2004). The variation of findings in multiple species of cloned animals suggests that cloning has different consequences in different species and even within a species, warranting further study.

Consequences for Subsequent Generations

One proposed application of cloning is the production of animals with highly desirable traits, such as cat-

tle for high quality beef, or transgenic pigs for tissue replacement in humans. Once a founder line is established, generations of offspring exhibiting the same trait(s) would follow, either by cloning or natural mating. However, a major concern arises about the offspring of cloned animals, particularly in light of the high incidence of altered phenotypes exhibited by the first generation of clones.

Multiple generations of cloned animals have not been reliably generated in species other than the mouse (Wakayama et al., 2000). The success rate in generating clones from clones decreased with increasing generations, and the two independent lines ultimately generated mouse clones up to the fourth and sixth generations. It is interesting to note that, although these clones of clones are obese similar to the first generation clones, the phenotype was not *enhanced* in subsequent generations (Wakayama et al., 2000). That is, cloning of clones did not produce progressively larger mice.

In order to further ascertain whether clone phenotypes are a genetically heritable trait, various groups have studied offspring derived by mating cloned males with cloned females. Phenotypes such as obesity, aberrant methylation patterns, placental hypertrophy, and open eyelids at birth were not transmitted to offspring when clones were mated with other cloned mice or with wild-type mice (Shimozawa et al., 2002; Tamashiro et al., 2002; Heyman et al., 2004; Wells et al., 2004). Furthermore, there is one report that an F3 generation also does not display the phenotype of the original clones (Shimozawa et al., 2002).

Similarly, viability of offspring from cloned cattle is significantly improved, compared to the cloned generation and comparable to conventional livestock (Wells et al., 2004). While the cloned generation had abnormal serum biochemistry and hematology parameters, and experienced more bouts of illness than usual, the same blood measures in the offspring of clones were within normal ranges and the inci-

dence of illness was negligible (Wells et al., 2004). Although there are limited numbers of offspring from cloned cattle spanning a wide range of ages, there have been no deaths among this group, in contrast to constant annual mortality reported in the cloned generation (Wells et al., 2004).

Epigenetic alterations can cause abnormal imprinting and/or reprogramming that alters gene expression and consequently the phenotype in clones. However, it appears that these alterations may be corrected during gametogenesis, thus producing offspring that do not exhibit the same phenotype as their cloned parents. Together, these studies suggest that an epigenetic mechanism may be responsible for some of the altered phenotypes exhibited by cloned animals, since the phenotypes are not transmitted to their offspring. There is evidence that cloning and artificial embryo culture environments, such as those commonly used in ART, can affect the expression of imprinted genes through epigenetic modifications during early development (Young and Fairburn, 2000). This raises important questions about the short- and long-term consequences of various forms of ART on offspring.

Clinical Relevance

The general consensus from studies of cloned animals is that while nuclear transfer may produce apparently healthy offspring, it often leads to a wide spectrum of adverse conditions and mortality of embryos and fetuses. While some aberrations may be evident immediately, the results of several studies now suggest that abnormalities may not be manifest until adulthood and longitudinal studies of cloned offspring are required.

The variability of phenotypes in clones, even within a species (Cibelli et al., 2002), suggests that perhaps the nuclear transfer and cloning process is neither the specific nor the sole culprit. Many manipulations and processes involved in cloning are also involved in ART used in humans. These include oo-

TABLE 1. Summary of In Vitro Manipulations

	Natural mating	In vitro embryo culture	In vitro fertilization (IVF)	Intracytoplasmic sperm injection (ICSI)	Round spermatid injection (ROSI)	Clone
Fertilization method	In vivo	In vivo	In vitro	In vitro	In vitro	In vitro
In vitro mechanical manipulation	None	None	None	Microinject sperm head into oocyte	Microinject pre-sperm cell into oocyte	Microinject adult somatic cell nucleus into oocyte
Embryo culture	No	Yes	Yes	Yes	Yes	Yes
Embryo transfer	No	Yes	Yes	Yes	Yes	Yes
Effect	–	Embryo culture	In vitro fertilization	Microinjection of sperm	Use of pre-sperm cell	Use of differentiated somatic cell

cyte maturation, mechanical manipulation, oocyte activation, in vitro embryo culture, and embryo transfer, some of which have already been associated with increased birth defects and abnormalities in cloned animal offspring (van Wagtendonk-de Leeuw et al., 1998; Pace et al., 2002) and in human clinical studies (Hansen et al., 2002; Kurinczuk et al., 2004; Hansen et al., 2005).

LONG-TERM HEALTH CONSEQUENCES OF ASSISTED REPRODUCTIVE TECHNIQUES

A landmark event in the development of the assisted reproduction field was the first human baby produced by IVF (Steptoe and Edwards, 1978). Since then, significant advances have been made to increase the success rate of ART in humans (CDC, 2002), primarily due to improvements in IVF techniques and development of new techniques that circumvent infertility barriers. It is clear that the field has moved forward quickly. In 1992, Palermo et al. (1992) reported the birth of an apparently healthy baby after injection of a single spermatozoa from an infertile man directly into his wife's oocyte, a process generally called ICSI. Although Iritani and Hosoi (1989) previously reported the birth of ICSI offspring in the

rabbit, it was not until 1995 that ICSI was demonstrated in mice (Kimura and Yanagimachi, 1995a). Obviously, in the case of ICSI, the clinical application came before an animal model could prove its biological and medical safety.

ART bypasses various biological processes and interactions that occur with natural IVF (Yanagimachi, 2005). Although it is now well established that conception and development to full term can occur despite exclusion of these processes and interactions, the possible adverse consequences cannot be completely ruled out. Studies in humans have started but will take years to complete. In contrast, the influences of in vitro manipulations can be examined in rodents, domestic species, and perhaps nonhuman primates in a relatively short period of time depending on the species. Table 1 is a summary of common ART and the manipulations involved in each. The effect of embryo culture or IVF, for example, can be studied using different groups of animals. There are many questions that remain unanswered, and studies to ascertain the long-term effects of ART on offspring should be encouraged. The information obtained from longitudinal studies in animal models will be valuable in evaluating the safety of ART in humans.

IVF and ICSI

IVF is typically used in cases where sperm is unable to gain access to the oocyte in vivo. In the procedure, sperm and eggs are collected and mixed in vitro where fertilization occurs. ICSI, on the other hand, is a more drastic form of ART. It is typically used to counter male factor infertility characterized by abnormal sperm morphology or drastically decreased sperm count and/or sperm motility. The major concern with ICSI is that it also bypasses many natural selection mechanisms that sperm encounter during natural fertilization, since sperm are selected by the clinician by subjective criteria prior to injection into the oocyte.

Although most infants conceived after ART such as IVF an ICSI are apparently normal, they are more likely to be born prematurely and have lower birth weight, perhaps due to the high incidence of multiple births, than naturally conceived infants (Bergh et al., 1999; Hansen et al., 2002; Kallen et al., 2002; Ludwig and Katalinic, 2002; Schieve et al., 2002; Van Steirteghem et al., 2002; Reynolds et al., 2003). Data about birth defects in children conceived through ART are less clear and have generated conflicting results. For example, a study by Bowen et al. (1998) suggests that ICSI children exhibit neurological delays at one year of

age, while other studies subsequently reported that ICSI children have scores on tests of cognition that fall within normal range and are comparable to those of children conceived naturally (Sutcliffe et al., 1999). A recent report by Hansen et al. (2005) reviewed all papers published prior to March 2003 about the prevalence of birth defects in infants conceived following IVF or ICSI compared to spontaneously conceived infants. Pooled results from their study suggest that children born after ART are at an increased risk of having birth defects compared to spontaneously conceived controls. While these data suggest that ART-conceived infants may have a greater incidence of birth defects, the question remains about whether apparently normal infants and children conceived by this method will develop side effects at adolescence or in adulthood. There are limited prospective studies of the physical and mental development of ICSI offspring. It is important to point out that although these technologies have been used extensively in humans for a number of years, the oldest IVF human is still in her mid-20s, and the oldest ICSI human is only in his early teens. Thus the long-term effects, if they indeed exist, may not be manifest for many years.

ROSI

Some infertility conditions prevent development of mature sperm, thus leading to the development of a technique to use prespermatozoal cells, or round spermatids, instead. ROSI was first developed in the mouse when live offspring were generated using round spermatids, first by electrofusion (Ogura et al., 1994) and later by microinjection (Kimura and Yanagimachi, 1995b). This procedure circumvents spermiogenesis in addition to all of the prefertilization events that are bypassed using ICSI. ROSI has been successful in humans (Tesarik and Mendoza, 1996; Tesarik et al., 1996; Barak et al., 1998); however, the overall success rate remains low (Levran et al., 2000).

The reasons for this may be attributed to the high incidence of chromosomal abnormalities in ROSI embryos (Benkhalifa et al., 2004), and thus the technique is not widely used in humans and needs to be studied further.

The possible behavioral consequences of ROSI were examined in the fifth generation of mice derived by sequential ROSI procedures. Fertility in ROSI offspring was normal, and behavioral tests of learning and memory did not reveal any abnormalities (Tamashiro et al., 1999). It is important to point out that normal, fertile mice were used in these studies. Therefore, while these results suggest that the ROSI procedure itself does not adversely affect behavior and learning or memory in resulting offspring, further behavioral studies using infertile models are required. Subsequent reports demonstrate that ROSI can be used to produce offspring in mice that are incapable of spermiogenesis (Yanagimachi et al., 2004), or harbor a mutation that results in deficits in spermatogenesis (Meng et al., 2002). However, beyond documentation that live, apparently healthy, offspring were born, the phenotype of mice generated by ROSI using these infertile mouse models was not studied.

Embryo Cryopreservation

Embryo cryopreservation is routinely used to preserve embryos for transfer at a later date. Mice that were produced from cryopreserved embryos were examined from birth through senescence. The mice that were cryopreserved had significantly higher body weights as adults and during senescence. Behavioral studies of these mice revealed that some of the cryopreserved mice were not as proficient as controls in a learning and memory task (Dulioust et al., 1995). In humans, Wennerholm et al. (1997), reported that embryo cryopreservation did not have an effect on growth and health through the first 18 months of life. However, since only a short

time period was examined, the authors did not rule out the possibility of the appearance of delayed adverse conditions during later childhood, adolescence, or in adulthood.

Note on In Vitro Handling of Gametes and Embryos

Abnormalities in offspring can result from exposure of eggs and embryos to in vitro culture conditions alone. Exposure to culture media can affect embryonic development and has long-term consequences since the media often contain chemicals the embryo is not normally exposed to or chemicals at nonphysiological concentrations. Embryos may additionally be deprived of chemical signals that they normally encounter while traversing the female reproductive tract, such as various growth factors. Recent studies in mice suggest that in vitro culture of in vivo fertilized embryos can have long-term consequences affecting development, behavior, and imprinted gene expression in offspring (Doherty et al., 2000; Ecker et al., 2004). Other studies revealed that mice cultured in vitro as embryos had higher body weights as adults compared to in vivo controls (Tamashiro et al., 2002; Sjoblom et al., 2005). The effect of suboptimal culture conditions (inclusion of fetal calf serum) also had a similar effect on body weight compared to mice cultured in standard culture medium (Fernandez-Gonzalez et al., 2004). Additionally, the mice displayed developmental and behavioral deficiencies. There were no in vivo produced controls included in this study; however, in light of other studies documenting increased body weight due to in vitro culture, it is possible that both groups of mice subject to in vitro embryo culture had higher body weights than in vivo-produced controls. This further suggests that inclusion (Fernandez-Gonzalez et al., 2004) or lack of (Sjoblom et al., 2005) specific components within the culture medium may be responsible for increased body weights in animals

exposed to artificial culture media as embryos.

IVF techniques are widely used in livestock production. This is because it is more efficient in improving genetic characteristics of herds and distributing genetic gain in livestock than relying on natural reproduction. The widespread use of ART in livestock has an additional benefit, i.e., of providing information about the effects of ART on the health and welfare of offspring. In cattle and sheep, in vitro embryo culture has been associated with increased birth weight or LOS (Thompson et al., 1995; Walker et al., 2000), abnormal physiology (Garry et al., 1996), and abnormal organ and skeletal development (Sinclair et al., 1999; Farin et al., 2001, Farin et al., 2004). At birth, placental abnormalities are also common (Kruip and den Daas, 1997; Sinclair et al., 1999), and perinatal mortality is higher than that of natural fertilization (Walker et al., 1992).

Finally, manipulation of gametes and embryos could have an impact on embryo development. The degree of cellular trauma and damage resulting from these processes to an egg, sperm, or embryo may also depend on the technical skill of the individual and the laboratory doing the manipulations (Menezo et al., 2000; Perry and Wakayama, 2002).

While animal models provide an indication of the influence that ART may have on resulting offspring, comprehensive prospective clinical studies with appropriate controls are essential in humans (Schatten et al., 1998; Niemitz and Feinberg, 2004). A growing body of literature exists concerning the health and development of children conceived through the use of ART, but many have conflicting results. The overall consensus appears to be that while there is no evidence at present that these children have significant deficiencies, longer term, prospective studies are warranted to determine whether these technologies have delayed adverse effects that may not be readily apparent. From another perspective, studies of ART effects on embryos and offspring

may shed light on the long-term influence of environmental factors during natural pregnancies as well.

CONCLUSION

In this review, we have highlighted some of the adverse consequences that have been documented in cloned animals. In addition, we have discussed studies of the effects of ART in animal models and in humans. It is important to remember that although these techniques do sometimes produce abnormalities, the majority of offspring are born apparently normal and survive to adulthood. Additionally, we must emphasize that the effects of ART and cloning observed in animal models do not necessarily indicate that they will occur in humans. Furthermore, there is inherent variation in the technical aspects of these procedures that will be introduced by individual laboratories as well as individual technicians. Studies of the consequences of cloning and ART on offspring must be conducted in a highly responsible and sensitive manner. Investigators must realize that the findings in these studies have far reaching consequences, and must understand the ultimate impact of their studies. There are roughly one million humans worldwide that have already been conceived by various forms of ART since 1978 (Powell, 2003). While it is clear that the use of ART to treat human infertility is increasing, the clinical procedures used in ART are rapidly outpacing the underlying science. While it appears that various ART are not innocuous, conclusions about its overall safety cannot be made until more systematic and prospective studies are conducted. Somatic cell cloning can be considered the most extreme form of ART, and its use in human reproduction must be curtailed until much more is known about its mechanisms and safety for resulting offspring. Likewise, its use in other applications, such as producing pharmaceuticals and replacement tissues and organs for humans, needs to be examined thoroughly prior to extension into clinical use.

REFERENCES

Aldhous P. 2000. The cloned mouse that roared is silenced. Nature 405:268.

Barak Y, Kogosowski A, Goldman S, et al. 1998. Pregnancy and birth after transfer of embryos that developed from single-nucleated zygotes obtained by injection of round spermatids into oocytes. Fertil Steril 70:67–70.

Benkhalifa M, Kahraman S, Biricik A, et al. 2004. Cytogenetic abnormalities and the failure of development after round spermatid injections. Fertil Steril 81:1283–1288.

Bergh T, Ericson A, Hillensjo T, et al. 1999. Deliveries and children born after in-vitro fertilisation in Sweden 1982–95: a retrospective cohort study. Lancet 354:1579–1585.

Betts D, Bordignon V, Hill J, et al. 2001. Reprogramming of telomerase activity and rebuilding of telomere length in cloned cattle. Proc Natl Acad Sci USA 98:1077–1082.

Blasco MA. 2002. Immunosenescence phenotypes in the telomerase knockout mouse. Springer Semin Immunopathol 24:75–85.

Bowen JR, Gibson FL, Leslie GI, et al. 1998. Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. Lancet 351:1529–34.

CDC. 2002. Assisted reproductive technology success rates. National summary and fertility clinics reports. National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention. Available at: http://www.cdc.gov/reproductivehealth/ART02/index.htm. Accessed May 18, 2005.

Chavatte-Palmer P, Heyman Y, Richard C, et al. 2002. Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 66:1596–1603.

Chavatte-Palmer P, Remy D, Cordonnier N, et al. 2004. Health status of cloned cattle at different ages. Cloning Stem Cells 6:94–100.

Chesne P, Adenot PG, Viglietta C, et al. 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol 20:366–369.

Cho JH, Kimura H, Minami T, et al. 2001. DNA methylation regulates placental lactogen I gene expression. Endocrinology 142:3389–3396.

Cibelli JB, Stice SL, Golueke PJ, et al. 1998. Cloned transgenic calves pro-

duced from nonquiescent fetal fibroblasts. Science 280:1256-1258.

Cibelli JB, Campbell KH, Seidel GE, et al. 2002. The health profile of cloned animals. Nat Biotechnol 20:13-14.

Doherty AS, Mann MR, Tremblay KD, et al. 2000. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod 62:1526-1535.

Dulioust E, Toyama K, Busnel MC, et al. 1995. Long-term effects of embryo freezing in mice. Proc Natl Acad Sci USA 92:589-593.

Ecker DJ, Stein P, Xu Z, et al. 2004. Long-term effects of culture of preimplantation mouse embryos on behavior. Proc Natl Acad Sci USA 101:1595-1600.

Edwards L, Peura T, Hartwich K, et al. 2002. Postnatal growth and circulating ACTH and cortisol concentrations during the first month of life in cloned lambs. Endocrinology 143:3699-3702.

Eggan K, Akutsu H, Loring J, et al. 2001. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci USA 98:6209-6214.

Evans MJ, Gurer C, Loike JD, et al. 1999. Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep. Nat Genet 23:90-93.

Farin PW, Crosier AE, Farin CE. 2001. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 55:151-170.

Farin CE, Farin PW, Piedrahita JA. 2004. Development of fetuses from in vitro-produced and cloned bovine embryos. J Anim Sci 82(E-Suppl): E53–E62.

Fernandez-Gonzalez R, Moreira P, Bilbao A, et al. 2004. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci USA 101:5880-5885.

Fox WM. 1965. Reflex-ontogeny and behavioural development of the mouse. Anim Behav 13:234-241.

Galli C, Lagutina I, Crotti G, et al. 2003. Pregnancy: a cloned horse born to its dam twin. Nature 424:635.

Garry FB, Adams R, McCann JP, et al. 1996. Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 45:141-152.

Giles J, Knight J. 2003. Dolly's death leaves researchers woolly on clone ageing issue. Nature 421:776.

Govoni KE, Tian XC, Kazmer GW, et al. 2002. Age-related changes of the somatotropic axis in cloned Holstein calves. Biol Reprod 66:1293-1298.

Hales CN, Barker DJ. 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35:595-601.

Hansen M, Kurinczuk JJ, Bower C, et al. 2002. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med 346:725-730.

Hansen M, Bower C, Milne E, et al. 2005. Assisted reproductive technologies and the risk of birth defects—a systematic review. Hum Reprod 20:328-338.

Heyman Y, Richard C, Rodriguez-Martinez H, et al. 2004. Zootechnical performance of cloned cattle and offspring: preliminary results. Cloning Stem Cells 6:111-120.

Holt WV, Pickard AR, Prather RS. 2004. Wildlife conservation and reproductive cloning. Reproduction 127:317-324.

Inoue K, Kohda T, Lee J, et al. 2002. Faithful expression of imprinted genes in cloned mice. Science 295:297.

Iritani A, Hosoi Y. 1989. Microfertilization by various methods in mammalian species. Prog Clin Biol Res 294:145-149.

Jiang L, Carter DB, Xu J, et al. 2004. Telomere lengths in cloned transgenic pigs. Biol Reprod 70:1589-1593.

Kallen B, Olausson PO, Nygren KG. 2002. Neonatal outcome in pregnancies from ovarian stimulation. Obstet Gynecol 100:414-419.

Kato Y, Tani T, Sotomaru Y, et al. 1998. Eight calves cloned from somatic cells of a single adult. Science 282:2095-2098.

Keefer CL, Baldassarre H, Keyston R, et al. 2001. Generation of dwarf goat (Capra hircus) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and in vitro-matured oocytes. Biol Reprod 64:849-856.

Keefer CL, Keyston R, Lazaris A, et al. 2002. Production of cloned goats after nuclear transfer using adult somatic cells. Biol Reprod 66:199-203.

Kimura Y, Yanagimachi R. 1995a. Intracytoplasmic sperm injection in the mouse. Biol Reprod 52:709-720.

Kimura Y, Yanagimachi R. 1995b. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 121:2397-2405.

Kruip TA, den Daas JH. 1997. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 47:43-52.

Kubota C, Tian XC, Yang X. 2004. Serial bull cloning by somatic cell nuclear transfer. Nat Biotechnol 22:693-694.

Kurinczuk JJ, Hansen M, Bower C. 2004. The risk of birth defects in children born after assisted reproductive technologies. Curr Opin Obstet Gynecol 16:201-209.

Lai L, Kolber-Simonds D, Park KW, et al. 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295:1089-1092.

Lanza RP, Cibelli JB, Blackwell C, et al. 2000. Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. Science 288:665-669.

Lanza RP, Cibelli JB, Faber D, et al. 2001. Cloned cattle can be healthy and normal. Science 294:1893-1894.

Lee JW, Wu SC, Tian XC, et al. 2003. Production of cloned pigs by whole-cell intracytoplasmic microinjection. Biol Reprod 69:995-1001.

Levran D, Nahum H, Farhi J, et al. 2000. Poor outcome with round spermatid injection in azoospermic patients with maturation arrest. Fertil Steril 74:443-449.

Ludwig M, Katalinic A. 2002. Malformation rate in fetuses and children conceived after ICSI: results of a prospective cohort study. Reprod Biomed Online 5:171-178.

Matsuzaki M, Shiga K. 2002. Endocrine characteristics of cloned calves. Cloning Stem Cells 4:261-267.

Menezo YJ, Veiga A, Pouly JL. 2000. Assisted reproductive technology (ART) in humans: facts and uncertainties. Theriogenology 53:599-610.

Meng X, Akutsu H, Schoene K, et al. 2002. Transgene insertion induced dominant male sterility and rescue of male fertility using round spermatid injection. Biol Reprod 66:726-734.

Miyashita N, Shiga K, Yonai M, et al. 2002. Remarkable differences in telomere lengths among cloned cattle derived from different cell types. Biol Reprod 66:1649-1655.

Morris RGM. 1981. Spatial localization does not require the presence of local cues. Learn Motiv 12:239-260.

Niemitz EL, Feinberg AP. 2004. Epigenetics and assisted reproductive technology: a call for investigation. Am J Hum Genet 74:599-609.

Ogonuki N, Inoue K, Yamamoto Y, et al. 2002. Early death of mice cloned from somatic cells. Nat Genet 30:253-254.

Ogura A, Matsuda J, Yanagimachi R. 1994. Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci USA 91:7460-7462.

Ogura A, Inoue K, Takano K, et al. 2000. Birth of mice after nuclear transfer by electrofusion using tail tip cells. Mol Reprod Dev 57:55-59.

Ogura A, Inoue K, Ogonuki N, et al. 2002. Phenotypic effects of somatic cell cloning in the mouse. Cloning Stem Cells 4:397-405.

Pace MM, Augenstein ML, Betthauser JM, et al. 2002. Ontogeny of cloned cattle to lactation. Biol Reprod 67:334-339.

Palermo G, Joris H, Devroey P, et al. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340:17-18.

Perry AC, Wakayama T. 2002. Untimely ends and new beginnings in mouse cloning. Nat Genet 30:243-244.

Polejaeva IA, Chen SH, Vaught TD, et al. 2000. Cloned pigs produced by nu-

clear transfer from adult somatic cells. Nature 407:86–90.

Powell K. 2003. Fertility treatments: seeds of doubt. Nature 422:656–658.

Renard JP, Chastant S, Chesne P, et al. 1999. Lymphoid hypoplasia and somatic cloning. Lancet 353:1489–1491.

Reynolds MA, Schieve LA, Martin JA, et al. 2003. Trends in multiple births conceived using assisted reproductive technology, United States, 1997–2000. Pediatrics 111(5 Part 2):1159–1162.

Savage AF, Maull J, Tian XC, et al. 2003. Behavioral observations of adolescent Holstein heifers cloned from adult somatic cells. Theriogenology 60:1097–1110.

Schatten G, Hewitson L, Simerly C, et al. 1998. Cell and molecular biological challenges of ICSI: ART before science? J Law Med Ethics 26:29–37, 3.

Schatten G, Prather R, Wilmut I. 2003. Cloning claim is science fiction, not science. Science 299:344.

Schieve LA, Meikle SF, Ferre C, et al. 2002. Low and very low birth weight in infants conceived with use of assisted reproductive technology. N Engl J Med 346:731–737.

Shay JW, Wright WE. 2000. Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol 1:72–76.

Shiels PG, Kind AJ, Campbell KH, et al. 1999. Analysis of telomere lengths in cloned sheep. Nature 399:316–7.

Shimozawa N, Ono Y, Kimoto S, et al. 2002. Abnormalities in cloned mice are not transmitted to the progeny. Genesis 34:203–207.

Shin T, Kraemer D, Pryor J, et al. 2002. A cat cloned by nuclear transplantation. Nature 415:859.

Sinclair KD, McEvoy TG, Maxfield EK, et al. 1999. Aberrant fetal growth and development after in vitro culture of sheep zygotes. J Reprod Fertil 116:177–86.

Singh U, Fohn LE, Wakayama T, et al. 2004. Different molecular mechanisms underlie placental overgrowth phenotypes caused by interspecies hybridization, cloning, and Esx1 mutation. Dev Dyn 230:149–164.

Sjoblom C, Roberts CT, Wikland M, et al. 2005. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. Endocrinology 146(5):2142–2152.

Steptoe PC, Edwards RG. 1978. Birth after the reimplantation of a human embryo. Lancet 2(8085):366.

Sutcliffe AG, Taylor B, Li J, et al. 1999. Children born after intracytoplasmic sperm injection: population control study. BMJ 318:704–705.

Tamashiro KL, Kimura Y, Blanchard RJ, et al. 1999. Bypassing spermiogenesis for several generations does not have detrimental consequences on the fertility and neurobehavior of off-spring: a study using the mouse. J Assist Reprod Genet 16:315–324.

Tamashiro KL, Wakayama T, Blanchard RJ, et al. 2000. Postnatal growth and behavioral development of mice cloned from adult cumulus cells. Biol Reprod 63:328–334.

Tamashiro KL, Wakayama T, Akutsu H, et al. 2002. Cloned mice have an obese phenotype not transmitted to their offspring. Nat Med 8:262–267.

Tamashiro KL, Wakayama T, Yamazaki Y, et al. 2003. Phenotype of cloned mice: Development, behavior and physiology. Exp Biol Med (Maywood) 228:1193–1200.

Tamashiro KL, Sakai RR, Yamazaki Y, et al. 2005. Health consequences of cloning mice. In: Inui A, editor. Health consequences of cloning. Taylor & Francis. Boca Raton, FL. pp. 1–16.

Tanaka S, Oda M, Toyoshima Y, et al. 2001. Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. Biol Reprod 65:1813–1821.

Tesarik J, Mendoza C. 1996. Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. Hum Reprod 11:772–779.

Tesarik J, Rolet F, Brami C, et al. 1996. Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. Hum Reprod 11:780–783.

Thompson JG, Gardner DK, Pugh PA, et al. 1995. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod 53:1385–1391.

Tian XC, Xu J, Yang X. 2000. Normal telomere lengths found in cloned cattle. Nat Genet 26:272–273.

Van Steirteghem A, Bonduelle M, Devroey P, et al. 2002. Follow-up of children born after ICSI. Hum Reprod Update 8:111–116.

van Wagtendonk-de Leeuw AM, Aerts BJ, den Daas JH. 1998. Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study. Theriogenology 49:883–894.

Vogel G. 2001. Reproductive biology: cloning: could humans be next? Science 291:808–809.

Wakayama T, Perry AC, Zuccotti M, et al. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369–374.

Wakayama T, Yanagimachi R. 1999. Cloning of male mice from adult tail-tip cells. Nat Genet 22:127–128.

Wakayama T, Rodriguez I, Perry AC, et al. 1999. Mice cloned from embryonic stem cells. Proc Natl Acad Sci USA 96:14984–14989.

Wakayama T, Shinkai Y, Tamashiro KL, et al. 2000. Cloning of mice to six generations. Nature 407:318–319.

Wakayama T, Yanagimachi R. 2001. Mouse cloning with nucleus donor cells of different age and type. Mol Reprod Dev 58:376–383.

Walker SK, Heard TM, Seamark RF. 1992. In vitro culture of sheep embryos without co-culture: successes and perspectives. Theriogenology 37:111–126.

Walker SK, Hartwich KM, Robinson JS. 2000. Long-term effects on offspring of exposure of oocytes and embryos to chemical and physical agents. Hum Reprod Update 6:564–577.

Wells DN, Forsyth JT, McMillan V, et al. 2004. The health of somatic cell cloned cattle and their offspring. Cloning Stem Cells 6:101–110.

Wennerholm UB, Hamberger L, Nilsson L, et al. 1997. Obstetric and perinatal outcome of children conceived from cryopreserved embryos. Hum Reprod 12:1819–1825.

Westhusin ME, Burghardt RC, Ruglia JN, et al. 2001. Potential for cloning dogs. J Reprod Fertil Suppl 57:287–293.

Westhusin M, Hinrichs K, Choi YH, et al. 2003. Cloning companion animals (horses, cats, and dogs). Cloning Stem Cells 5:301–317.

Wilmut I. 2002. Are there any normal cloned mammals? Nat Med 8:215–216.

Wilmut I, Paterson L. 2003. Somatic cell nuclear transfer. Oncol Res 13:303–307.

Wilmut I, Schnieke AE, McWhir J, et al. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–3.

Woods GL, White KL, Vanderwall DK, et al. 2003. A mule cloned from fetal cells by nuclear transfer. Science 301:1063.

Yanagimachi R. 2002. Cloning: experience from the mouse and other animals. Mol Cell Endocrinol 187:241–248.

Yanagimachi R, Wakayama T, Kishikawa H, et al. 2004. Production of fertile offspring from genetically infertile male mice. Proc Natl Acad Sci USA 101:1691–1695.

Yanagimachi R. 2005. Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. Reprod Biomed Online 10:247–288.

Young LE, Sinclair KD, Wilmut I. 1998. Large offspring syndrome in cattle and sheep. Rev Reprod 3:155–163.

Young LE, Fairburn HR. 2000. Improving the safety of embryo technologies: possible role of genomic imprinting. Theriogenology 53:627–648.

Young LE. 2001. Imprinting of genes and the Barker hypothesis. Twin Res 4:307–317.

Young LE, Fernandes K, McEvoy TG, et al. 2001. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 27:153–154.

Zhou Q, Renard JP, Le Friec G, et al. 2003. Generation of fertile cloned rats by regulating oocyte activation. Science 302:1179.

In vivo cell electrofusion

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Abstract

In vitro electrofusion of cells brought into contact and exposed to electric pulses is an established procedure. Here we report for the first time the occurrence of fusion of cells within a tissue exposed in vivo to permeabilizing electric pulses. The dependence of electrofusion on the ratio of applied voltage to distance between the electrodes, and thus on the achievement of in vivo cell electropermeabilization (electroporation) is demonstrated in the metastasizing B16 melanoma tumor model. The kinetics of the morphological changes induced by cell electrofusion (appearance of syncytial areas or formation of giant cells) are also described, as well as the kinetics of mitosis and cell death occurrence. Finally, tissue dependence of in vivo cell electrofusion is reported and discussed, since electrofusion has been observed neither in liver nor in another tumor type. Particular microenvironmental conditions, such as the existence of reduced extracellular matrices, could be necessary for electrofusion achievement. Since biomedical applications of in vivo cell electropermeabilization are rapidly developing, we also discuss the influence of cell electrofusion on the efficacy of DNA electrotransfer for gene therapy and of antitumor electrochemotherapy, in which electrofusion could be an interesting advantage to treat metastasizing tumors. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Electropermeabilization; Electroporation; Electrofusion; Electrochemotherapy; Cell fusion; Melanoma; In vivo; Normal tissue

1. Introduction

Spontaneous cell fusion among animal cells occurs during certain stages of the developmental program, like in the case of the generation of muscle fibers by myoblast fusion. Otherwise, cells in tissues do not fuse in spite of close contacts between them. There are two main reasons for the absence of cell fusion in tissues. First, the external matrix of the cells constitutes a physical obstacle to the fusion. Second, the structure of the lipid bilayers, with external hydrophilic layers at both sides of the internal hydrophobic core, prevents the spontaneous fusion of intact lipid bilayers. However, fusion between the plasma membrane and the membrane of cell internal vesicles, as well as fusion between membranes of different internal vesicles, or fission (e.g., at Golgi tubular networks), constantly occur in the living eucaryotic cells. Indeed, fusion and fission sustain membrane traffic, endocytosis and exocytosis, as well as intracellular transport in general. The

mechanisms underlying the 'internal' fusions and fissions are still under analysis: vesicle fusion occurs mainly on the basis of SNAREs proteins interactions [1], but other proteins, like the SM proteins or the Rab proteins might also be involved (reviewed in [2]). These proteins can also directly contribute to membrane fusion [1], like the spike protein of murine coronaviruses [3] or other viral proteins [2]. In spite of rapid accumulation of new results, the membrane fusion mechanisms are not yet fully described. Recently the role of the lipids has been pointed out because membrane fission of Golgi tubular networks has been shown to depend also on the acylation of lysophosphatidic acid by specific proteins [4]. However, the mechanisms underlying the fusion of intact plasma membranes of two different cells, like in the case of the myoblast, are still unknown at the molecular level.

In vitro cell–cell fusion can be induced artificially in cells that are in contact by adding to the cell culture medium either a fusogenic agent such as polyethylene glycol (PEG) [5] or a fusogenic virus, such as the Sendai virus [6] or by subjecting the cells to one or few electric pulses (EP). This last approach, termed electrofusion, is based on cell electropermeabilization [7] (also termed electroporation), which is caused by the increase in transmembrane poten-

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tial imposed by the application to the cells of external electric fields [8]. Reversible cell electropermeabilization corresponds to the transient and reversible modification of the plasma membrane structure [9]. In vitro, this modification has several biological consequences as well as some biotechnological and biomedical applications [10,11]: (i) increase of membrane permeability to non-permeant or low-permeant molecules, (ii) electroinsertion into the membrane of proteins possessing transmembrane domains, or (iii) electrofusion of cells.

In vitro electrofusion of cells growing in suspension is a common procedure for obtaining hybridomas [12–14]. Electrofusion of cells that require their attachment to a solid substrate to grow was already described [15–18]. Electrofusion occurs provided that the electropermeabilized membranes on the cells are brought to close contact [7,19,20]. It was also shown that in vitro the completion of the process requires several hours and leads to large rearrangements of cytoskeleton [21].

Ex vivo and in vivo electrofusion of human cells to rabbits cornea, obtained by the simultaneous application of the electric pulses and a mechanical pressure, was reported in 1989 and 1990 by Grasso and Heller [22–24]. It is noteworthy that cell electropermeabilization is easily achieved using appropriate EP [25–28] and that it has two interesting biomedical applications, electrochemotherapy [11,29,30] and DNA electrotransfer for gene therapy [31–33].

Here we report for the first time the occurrence of fusion of cells within a tissue exposed in vivo to permeabilizing EP. The kinetics of the morphological changes induced by cell electrofusion are also described. Finally we show and discuss the existence of a tissue dependence for the occurrence of cell electrofusion in vivo.

2. Materials and methods

2.1. Tumor cell culture and tumor production

B16 F0 melanoma cells (ATCC CRL 6322) were cultured in vitro using classical procedures and MEM culture medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 8% fetal calf serum (Gibco BRL). The LPB cell line is a clonal derivative of TBL.Cl2, a methylcholanthrene-induced C57Bl/6 mouse sarcoma cell line [34]. They were cultured under the same conditions than the B16 cells. Young (6–8 weeks) C57Bl/6 female mice were inoculated subcutaneously in the left flank with 1×10^6 to 1.2×10^6 syngeneic either B16 cells or LPB cells, producing tumors of 6–7 mm average diameter 8–10 days later. Every experimental situation, defined by the electrical conditions and the time of mouse sacrifice after the treatment, was performed in triplicate, that is, repeated using three mice. Mice were anesthetized using a mixture of xylazine 12.5

mg/kg (Bayer Pharma, Puteaux, France) and ketamine 125 mg/kg (Parke Davis, Courbevoie, France).

2.2. Tissue treatment with electric pulses

Tumor exposure to the EP was performed as previously described [35,36]. Briefly, stainless-steel external plate electrodes were placed on the both sides of the protruding tumor, contact with the skin being improved by means of electrocardiogram paste. Square-wave EP (8 pulses of 100 μs delivered at the frequency of 1 Hz) were generated by a PS 15 electropulsator (Jouan, St Herblain, France) and controlled through a VC-6025 oscilloscope (Hitachi, Japan). For 1350 V/cm pulses, 800 V were applied between two parallel electrodes 10 mm in size and 6 mm apart. For 2000 V/cm and 500 V/cm, 1200 and 300 V, respectively, were applied. After the treatment, mice were reinstalled in their cages for different periods (between 1 and 100 h) and then killed, and the tumors removed for histological processing.

For liver, a subxyphoid incision was done to expose the left lobe that was submitted to the EP. The same two plate electrodes used for tumors, also at a distance of 6 mm, were placed on both sides of the lobe. The square wave EP (100 μs, 1 Hz, 8 pulses) had an amplitude of 300 V (500 V/cm) or 600 V (1000 V/cm).

2.3. Histological procedures

Tumors and liver lobes were fixed in AFA (75% ethanol, 5% acetic acid and 2% of 40%-formaldehyde) for 24 h, dehydrated and embedded in paraffin. Slices of 5 μm were prepared using a Reichert–Jung 2030 microtome (Microm–Zeiss, Jena, Germany). Then slices were rehydrated and stained with 0.2% hemalun/0.3% eosin/5% safran (HES). The magnifications used are reported in the figures.

Slices were examined under a Leica DMRB (Leitz, Wetzlar, Germany) microscope equipped with an automatic photographic device. For each experimental condition, three mice were treated, and from each mouse, three tumor slices were prepared. All the slices were examined to detect the types of cell patterns present in each experimental condition. On slices prepared from the tumors of two out of three mice treated under the same conditions and killed after identical periods, the number of giant cells and of syncytial areas (see Section 3 for description) were counted, as well as the number of nuclei in each giant cell and in each syncytial area. The percentages of giant cells and of syncytial areas were calculated with respect to the total number of cells, while percentages of nuclei in giant cells and syncytial areas were determined with respect to the total number of nuclei. For that quantification, in each slice, the cells from three randomly chosen fields were counted. On the average, close to 1700 cells were counted for each experimental condition.
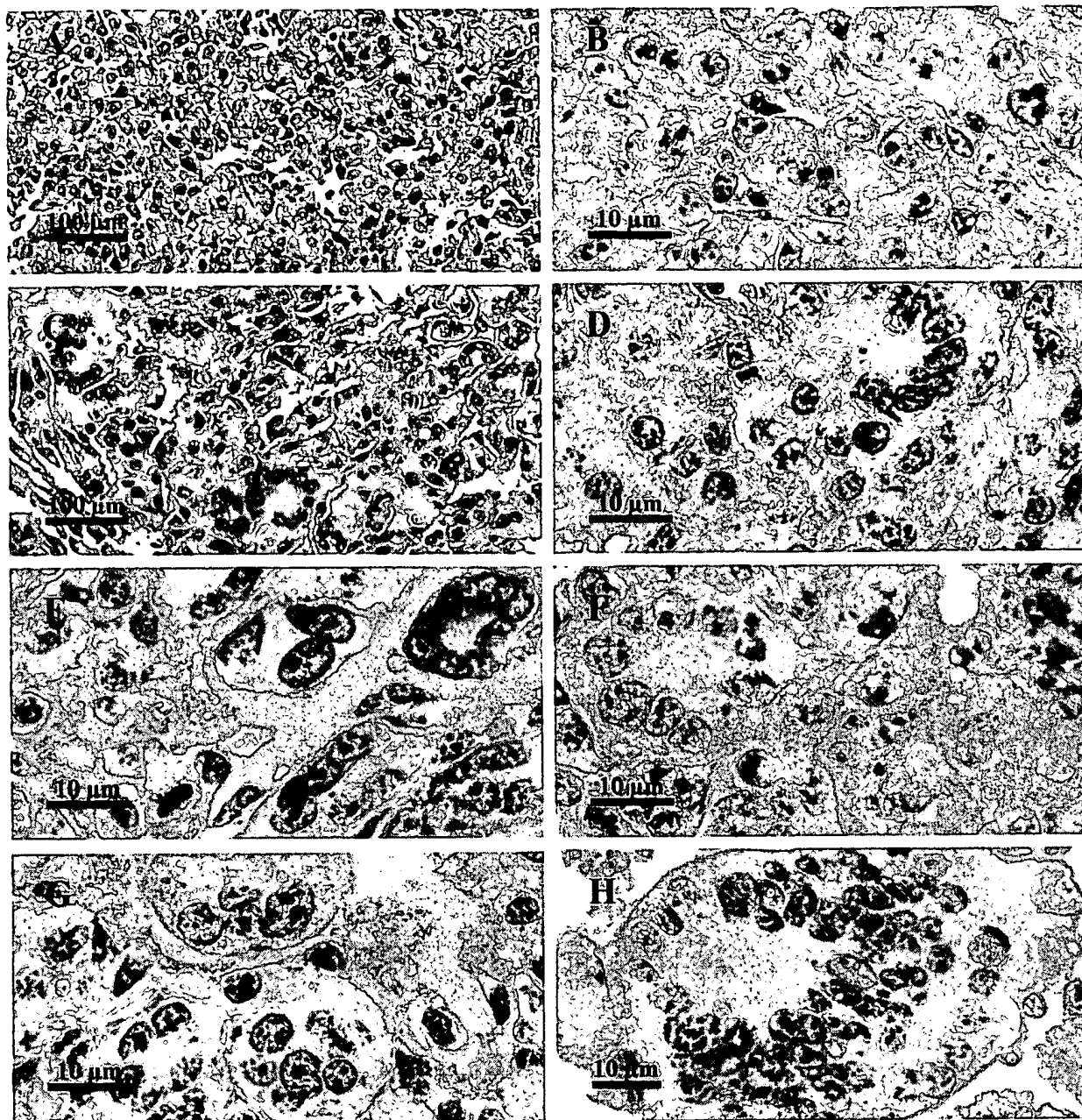
Fig. 1. Characteristic giant cells observed 70 h after the delivery of permeabilizing electric pulses to B16 tumors. B16 tumors were exposed to 8 EP of 1350 V/cm and 100 μs delivered at a frequency of 1 Hz (×1300). (A,B) Untreated control tumors fixed 70 h after the treatment of the treated tumors (A: ×130; B: ×1300). (C–G) Selected images of giant cells with a large cytoplasm and a peripheral ring of multiple nuclei (D–F) or multiple nuclei arranged randomly in the center (G) (C: ×130; D–G: ×1300). (H) A giant cell containing 64 nuclei (×750).

2.4. Determination of in vivo cell electropermeabilization

Bleomycin (Lab. Roger Bellon, Neuilly, France) was dissolved at 10 mg/ml in sterile 0.9% NaCl, and 100 μl of solution (approximately 50 mg/kg) was injected intravenously in the retro-orbitary sinus, 4 min before the electric pulse delivery. In electropermeabilized cells, this concentration led to chromatin condensation (pseudo-apoptosis), which was used as a marker. Mice were killed

5 h after EP delivery, and tumors and liver were immediately removed and processed for histological staining as described above.

2.5. Apoptosis-specific staining

Deparaffinated slices were washed in phosphate-buffered saline (PBS; 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 and 200 mM NaCl, pH 7.4), digested with 20 μg/ml Proteinase

K (Sigma, La Verpillière, France) and stained using the in situ cell death detection kit – alkaline phosphatase (Boehr-inger-Mannheim, Meylan, France), according to the instructions of the manufacturer.

3. Results

3.1. In vivo electrofusion

On the HES-stained histological slices of untreated control B16 tumors, most of the nuclei displayed a relatively regular size. They were usually located in the center of the tumor cell, symmetrically surrounded by cytoplasm (Fig. 1A,B). The membrane limiting each individual cell was clearly visible. On the contrary, in electrically treated tumors, 70 h after the delivery of permeabilizing EP, completely different patterns could be detected (Fig. 1C–H). The nuclei were reassembled in a common cytoplasm forming polynuclear giant cells. These giant cells contained several nuclei in close contact (a maximum of 64 nuclei was counted in one case, see Fig. 1H), organized in two different categories: (i) patterns with a large cytoplasm and a peripheral ring of multiple nuclei; (ii) patterns with multiple nuclei arranged randomly in the center. At 70 h after EP delivery (1350 V/cm), 4.8% of the nuclei (observed randomly as described in Section 2) were included in giant cells containing more than one nucleus. This was in clear contrast with the situation observed in the control tumors removed from the mice either at the time of the treatment of the treated tumors or 70 h later. Indeed in each of these control situations, out of the 1700 cells counted, no cell was found to contain more than one nucleus. Nevertheless, the complete examination of all the control sections showed that in controls, very few tumor cells did contain two nuclei.

3.2. Kinetics of fusion figures appearance

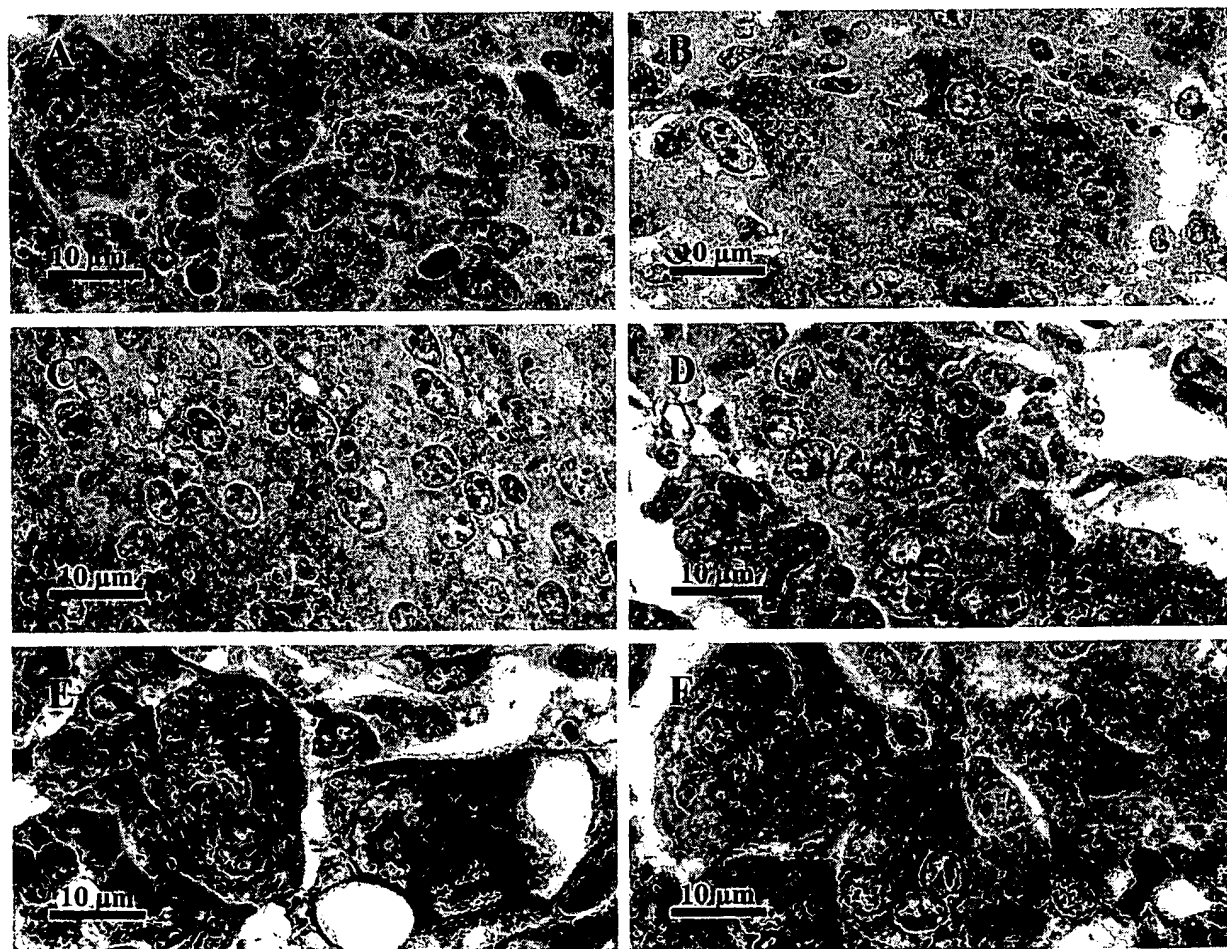Shortly after the treatment by the EP (1 h), no change in

Fig. 2. Morphological evolution of the fused cells after the delivery of permeabilizing electric pulses to B16 tumors. B16 tumors were treated as reported in Fig. 1. (A) Untreated control tumors fixed 70 h after the treatment of the treated tumors (×1300). (B–F) Figures observed in tumors fixed 3 h (B), 5 h (C), 10 h (D), 24 h (E) and 100 h (F) after EP delivery (×1300). At short times, large syncytial areas (B,C) as well as giant cells were observed. After 10 h, only characteristic giant cells (D–F) were detected.

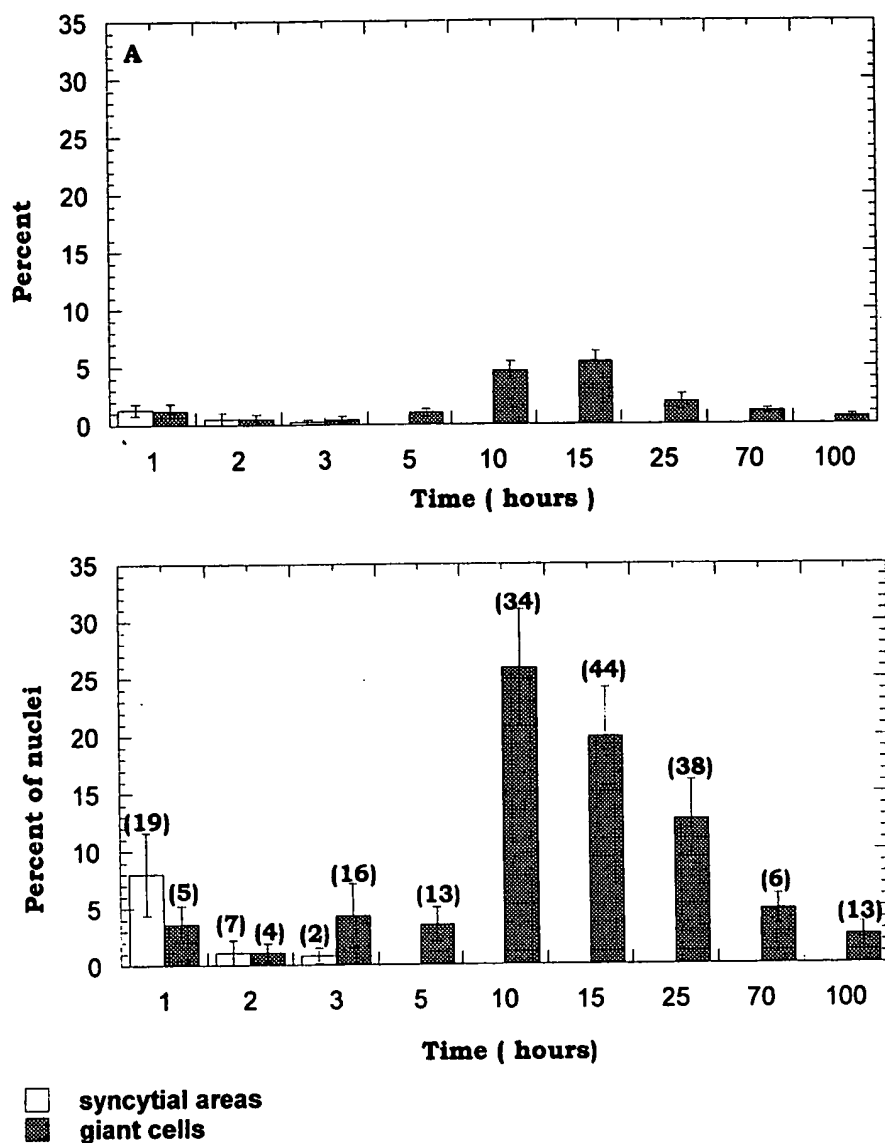H. Mekid, L.M. Mir / Biochimica et Biophysica Acta 1524 (2000) 118–130

Fig. 3. Kinetics of giant cell appearance after the delivery of permeabilizing electric pulses to B16 tumors. B16 tumors were treated as reported in Fig. 1. (A) Percentage of detectable syncytial areas and giant cells. (B) Percentage of nuclei included in syncytial areas and giant cells. Numbers in parentheses correspond to the maximum number of nuclei found in a single giant cell among those counted to establish the percentage of nuclei in giant cells.

the nuclei distribution was observed. However, two modifications were detected: the appearance of syncytial areas and the formation of giant cells. In the syncytial areas, the membranes separating the cells were no longer detectable (Fig. 2B,C). In the first hours after EP delivery, this modification was the predominant one. In the giant cells (Fig. 2D–F), the cytoplasm is clearly limited by a continuous membrane and the number of nuclei in each giant cell could be easily counted. Contrarily to the syncytial areas, giant cells were not abundant 1 h after tumor electric treatment.

One hour after EP delivery, the percentage of nuclei in the syncytial areas was already as high as 8%, and the percentage of syncytial areas reached 1.3%. In the giant

cells the percentage of the nuclei included was as high as 3.6%, but the percentage of the giant cells was equal to that observed for the syncytial areas (Fig. 3).

Two hours after EP delivery, the percentage of nuclei in both syncytial areas and giant cells as well as the percentage of syncytial areas and giant cells decreased and remained low for at least 3 more hours (Fig. 3A,B). Syncytial areas began to show massive cell death, detected by necrosis as well as by apoptotic changes in nucleus morphology. Finally, syncytial areas were no longer detected after 15 h. On the contrary, no decrease in the number of giant cells and in the number of nuclei per giant cell was observed after 3 and 5 h. Moreover, the limits of the fused cells became progressively more visible and easy to deter-
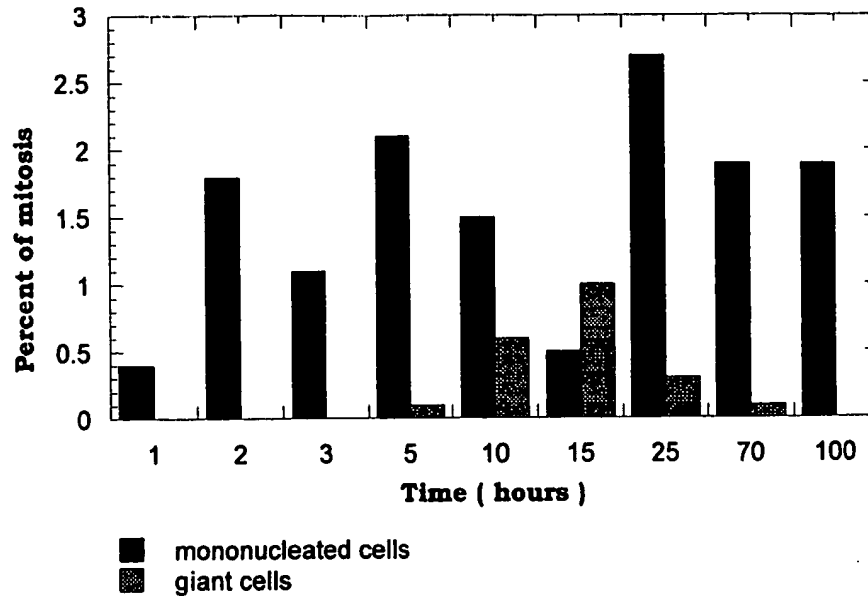
Fig. 4. Kinetics of mitosis occurrence in multinucleated fused cells. B16 tumors were treated as reported in Fig. 1.

mine. Later, the percentages of giant cells and of nuclei in giant cells sharply increased, reaching a maximum at 10 and 15 h (Fig. 3). In the microscopic fields where counts were performed, the number of fields containing giant cells also increased from 5 out of 18 at 3 h to 16 out of 18 at 15 h. In parallel (Fig. 3), the maximal number of nuclei per giant cell also increased after 5 h, to reach a maximum of 44 nuclei at 15 h (exceptionally, a single giant cell with a still higher number, 64 nuclei, was found at 70 h, as shown in Fig. 1H).

After the high percentages of giant cells and nuclei in them found at 10 and 15 h, a decrease was observed, leading to the presence of 2.5% of nuclei in giant cells and 0.6% of giant cells at 100 h after the treatment (Fig. 2F and Fig. 3). At this remote time after EP delivery, most of the giant cells contained only between three and six nuclei per cell.

3.3. Morphological changes within the giant cells

While no change in nuclei distribution was detected at 1 h after the electrical treatment, nuclei in giant cells were progressively found closer and closer at 3 h and later (Fig. 2). After 5 h, they began to display particular distributions resulting in patterns such as those shown in Fig. 1D–F. The cells in which the nuclei formed a circular pearl chain around the center of the cell were similar to those observed in vitro [37] and this nuclei pattern made the detection of the giant cells easy. This distribution became predominant 10h after the treatment. Pronounced eosinophilia of the cytoplasm also facilitated the detection of the fused cells since in general their cytoplasm staining was more homogeneous and more intense than that of the unfused cells.

3.4. Cell death and mitoses occurrence

Fig. 4 shows the percentages of mitoses observed in giant cells at various times after EP delivery. Most of these mitoses were abnormal (multipolar or irregular distribution of chromosomes). They were observed in both large and small multinucleated cells (Fig. 5A–C). Later on, mitoses were found only in the giant cells possessing a small-sized cytoplasm (thus probably containing a small number of nuclei) even at long times after the treatment. In syncytial areas, at the same time, no mitosis was observed.

Patterns that morphologically resembled apoptotic ones (chromatin condensation at the level of the nuclear envelope) were observed in the giant cells and in the syncytial areas at various times after the electric pulse delivery (Fig. 5D–F). At early times after the electric treatment these patterns were predominantly detected in the syncytial areas. In giant cells apoptotic patterns were detected at times longer than 15 h. Apoptosis was confirmed by immunostaining using an in situ cell death detection kit (Fig. 5G,H).

3.5. Dependence on cell electropermeabilization

In the B16 tumors exposed to electric pulses of only 500 V/cm, only the giant cells were observed (Fig. 6A,B,E,F) whereas no syncytial areas were found. Moreover, 15 h after EP delivery, giant cells contained only 4.8% of the counted nuclei while at 1350 V/cm giant cells contained 19.9% of the counted nuclei. The number of microscopic fields in which giant cells were detected, was also lower than at 1350 V/cm. For example, at 5 h, 3 out of 18 fields contained giant cells at 500 V/cm, compared to 7 out of 18
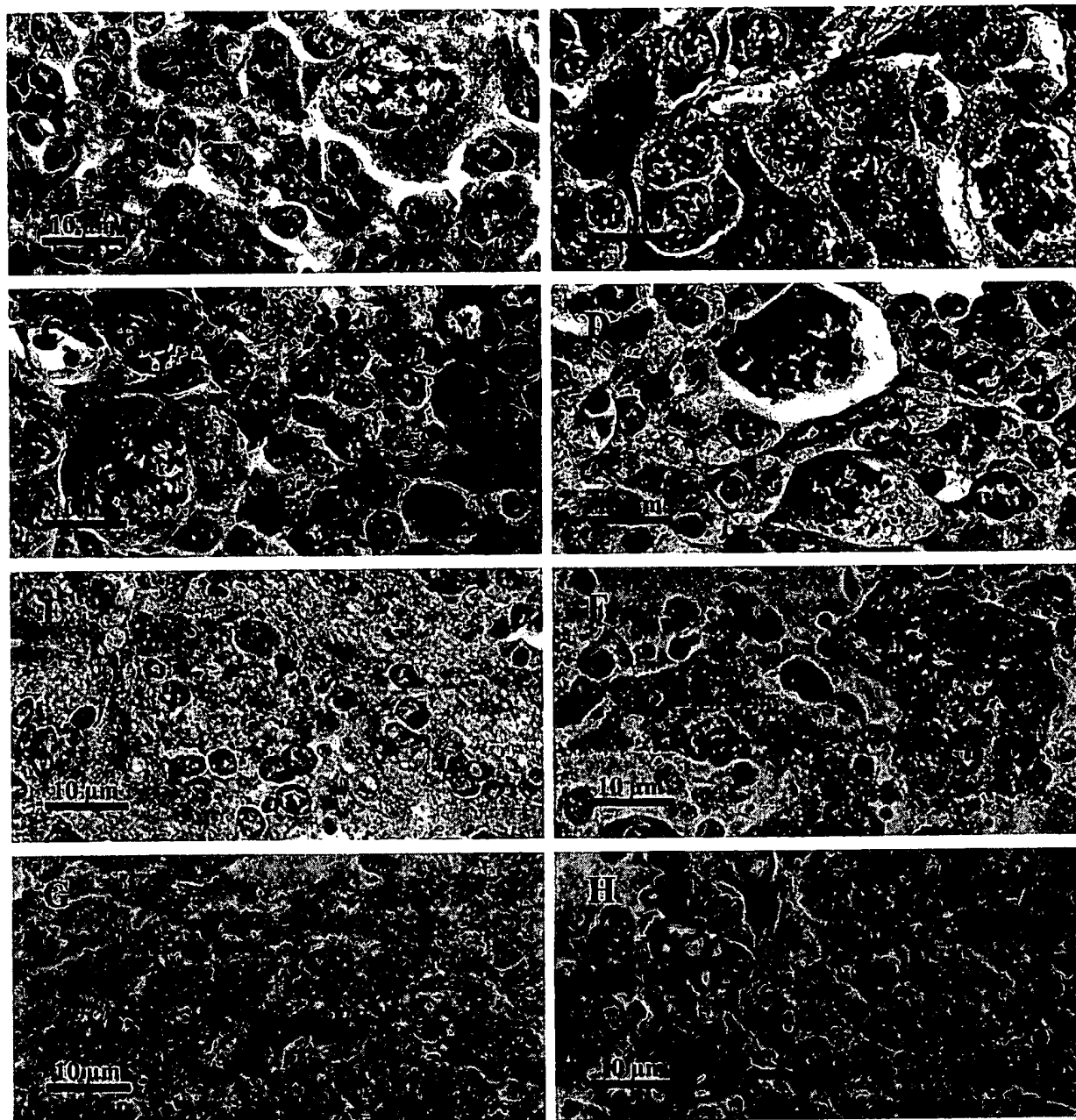
Fig. 5. Morphological aspects of mitosis and cell death (apoptosis) occurring in giant cells. B16 tumors were treated as reported in Fig. 1. (A–C) Mitotic patterns in giant cells at 10 h (A), 25 h (B) and 70 h (C) after EP delivery (×1300). (D) Mitosis and apoptosis occurring in two separate adjacent giant cells at 10 h after EP delivery (×1300). (E,F) Apoptotic patterns in giant cells at 25 h and 70 h after EP delivery (×1300). (G,H) Immunostained cells (using an in situ cell death detection kit) in an untreated control tumor (G) and in a tumor treated at 1350 V/cm, fixed at 25 h (H) (×1300).

at 1350 V/cm, and at 15 h, 9 out of 18 fields at 500 V/cm compared to 16 out of 18 at 1350 V/cm. The permeabilization control, made in parallel on tumors of mice previously injected with 1 mg of bleomycin, revealed a partial permeabilization of the tissue, essentially located in defined areas that could correspond to the location of the giant cells in tumors exposed only to EP (Fig. 6D), suggesting that fusion is indeed related to cell permeabilization, like in vitro [7].

3.6. Tissue dependence of the cell electrofusion

In the LPB fibrosarcomas, the tissue structure is more homogeneous than that of the B16 tumors. Individual cells can be easily identified, even though the plasma membranes are not always clearly visible (Fig. 7A). The LPB tumors were exposed to the same EP (same voltage to distance ratio, number of pulses, duration of each pulse and frequency of repetition, as well as identical electrode
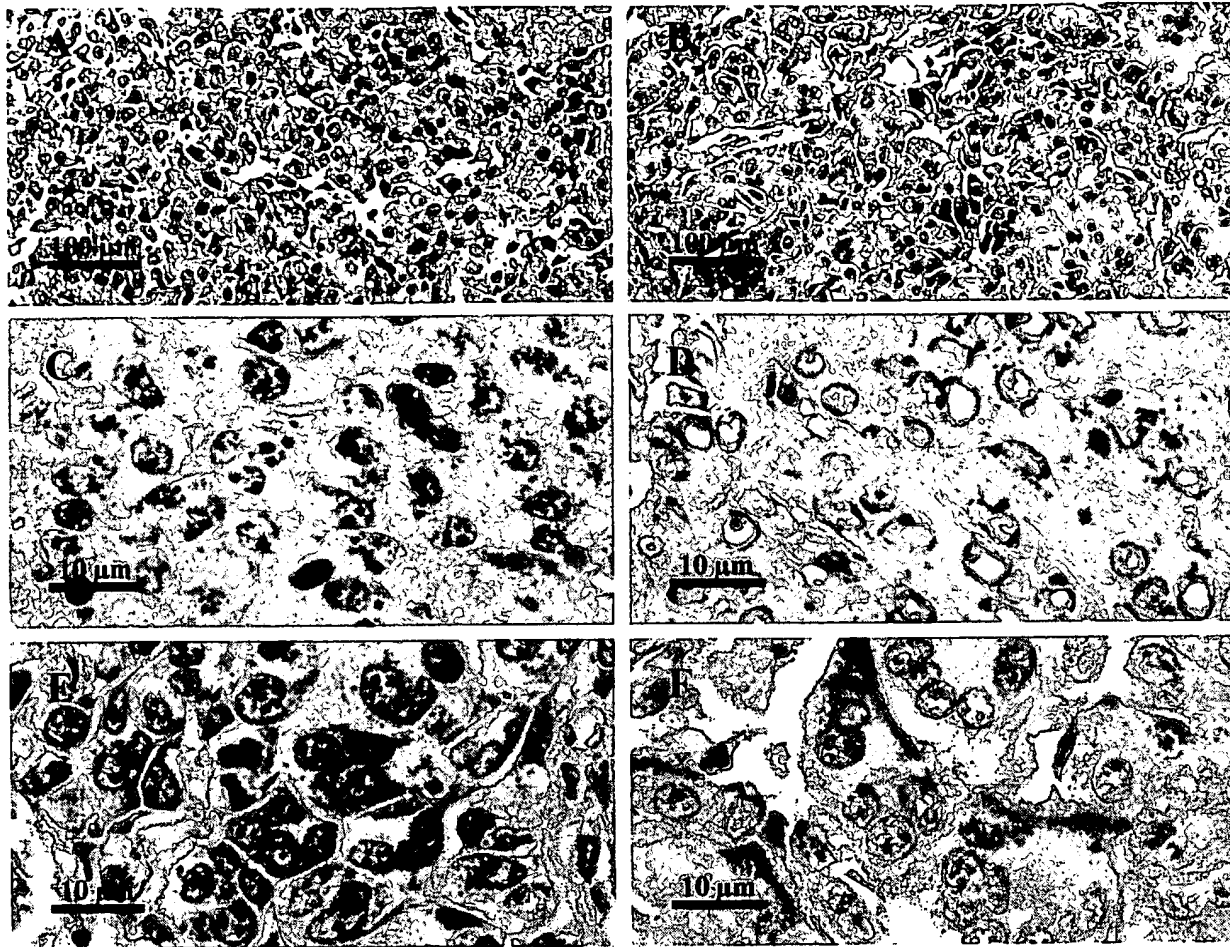
Fig. 6. Dependence of cell electrofusion on cell electropermeabilization in B16 tumors. B16 tumors were exposed to 8 EP of only 500 V/cm and 100 μs delivered at a frequency of 1 Hz, as described in Section 2. (A,C) Untreated control tumors fixed 70 h after the treatment of the treated tumors (A: ×130; C: ×1300). (B,E,F) Patterns observed in the small areas of the tumors displaying giant cells at 5 h (B,E) or at 15 h (F) after EP delivery (B: ×130; E,F: ×1300). (D) Pseudoapoptotic cells found, at 5 h after the electric treatment, in small areas of tumors of mice injected with 1 mg of bleomycin prior to EP delivery (×1300).

geometry) as those delivered to the B16 tumors. It was shown that these EP really permeabilized the LPB tumor cells, using bleomycin at a high dose as described above (Fig. 7B). Nevertheless, the electropermeabilizing EP did not result in the appearance of any of the particular figures observed in the B16 tumor slices. Indeed, panels C and D in Fig. 7 show that there were no morphological changes under the same conditions that lead to extensive electrofusion in B16 tumors. In order to be sure that the absence of cell electrofusion was not the result of a limited cell permeabilization (see above), higher voltages (2000 V/cm) were also delivered to the LPB fibrosarcomas. Even under these drastic permeabilization conditions, no electrofusion event was detected (Fig. 7E,F).

A similar search for fusion patterns was also performed in a normal murine tissue, the liver (Fig. 8). Liver tissue was chosen because it is a very homogeneous tissue in which histological analysis can be easily performed, and

because the amplitude of the electric pulses necessary to achieve a good permeabilization of the liver tissue cells was already determined in rabbits (Miklavcic, Šemrov, Mekid, Mir, submitted for publication) and in rats (Combettes, Tordjmann, Mir, unpublished results). In both rabbits and rats, EP of 500 V/cm to 600 V/cm have been found the most appropriate when using trains of 8 pulses of 100 μs at the frequency of 1 Hz delivered through parallel plates. Control of cell electropermeabilization under our experimental conditions in mice was done using the same test as for the determination of cell electropermeabilization in B16 and LPB tumors, i.e., using high doses of bleomycin injected 4 min before EP delivery (Fig. 8B). No figure corresponding to the electrofusion of hepatocytes was detected either at 5 h (Fig. 8C) or at 24 h after EP delivery (Fig. 8D). A further control was done by pulsing the liver at 1000 V/cm, and still no fusion was observed (Fig. 8E,F).
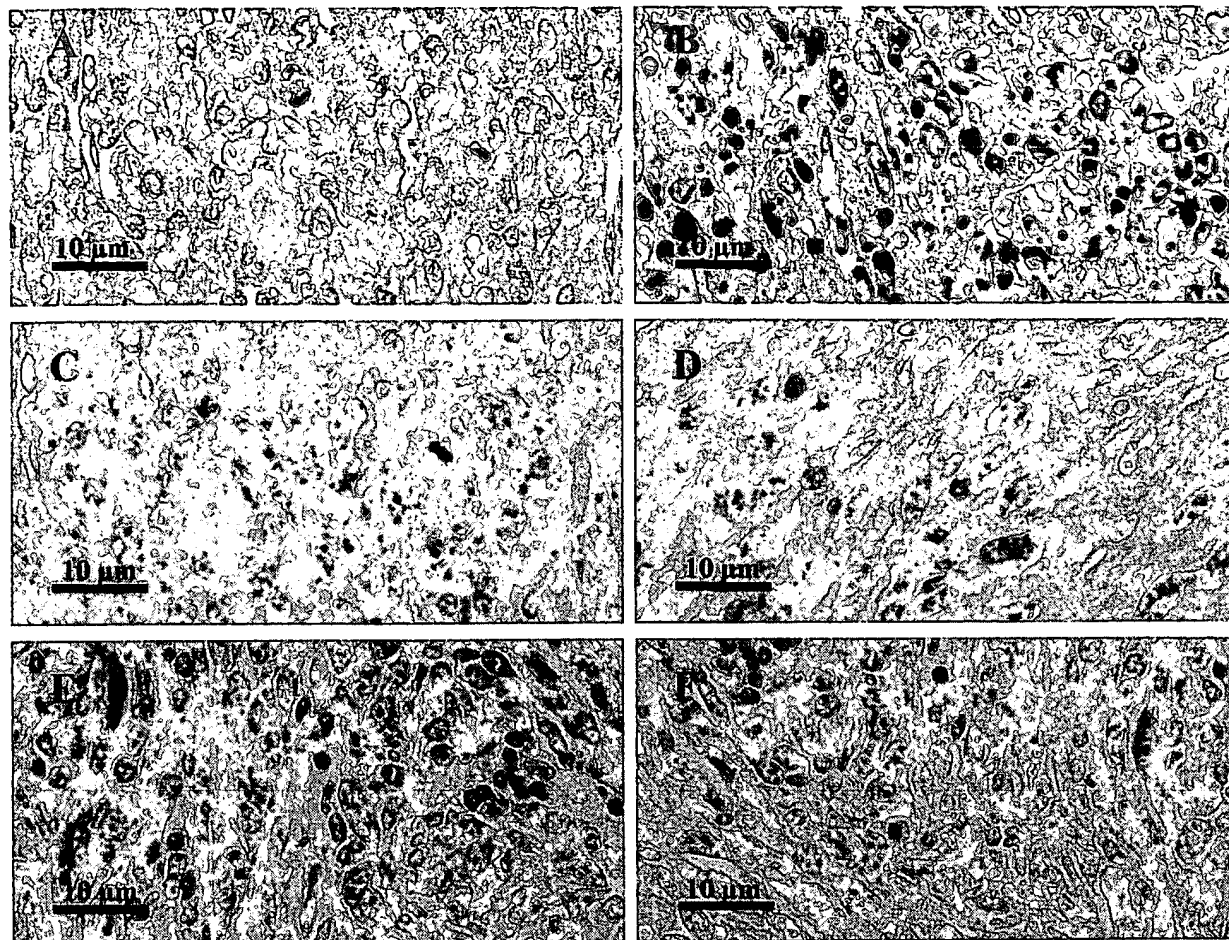
Fig. 7. Absence of electrofusion in the LPB fibrosarcoma after the delivery of permeabilizing electric pulses. LPB tumors were exposed to 8 EP of 100 μs and 1350 V/cm or 2000 V/cm, delivered at a frequency of 1 Hz, as described in Section 2. (A) An untreated control tumor fixed 24 h after the treatment of the treated tumors (×1300). (B) Cell electropermeabilization at 1350 V/cm as revealed by the presence of pseudoapoptotic cells found, at 5 h after the electric treatment, in small areas of tumors of mice injected with 1 mg of bleomycin prior to EP delivery (×1300). (C,D) Unfused cells of LPB tumors fixed 5 h (C) or 24 h (D) after electrical treatment at 1350 V/cm (×1300). (E,F) Unfused cells of LPB tumors fixed 5 h (E) or 24 h (F) after electrical treatment at 2000 V/cm (×1300).

4. Discussion

In the present work, we report the first observation of cell electrofusion in vivo, achieved in the B16 murine melanoma tissue when appropriate EP are delivered locally on the tumor.

In vivo electrofusion observed in B16 melanoma was quite unexpected because in our earlier work on the liver [38] and tumors such as LPB fibrosarcomas [35,36], we did not observe the cell fusion phenomenon comparable to the typical patterns shown in Fig. 1. In the light of the new results with B16 tumors, we have repeated the experiments on the LPB murine fibrosarcoma and on the normal liver of the syngeneic C57BL/6 mouse, this time carefully examining the possibility of cell fusion. In neither of these two tissues, cell electrofusion patterns were found.

Our observations are somehow reminiscent of the reports dealing with the electrofusion of human dispersed cells deposited in situ on the epithelium of rabbit cornea

and submitted to a concomitant mechanical pressure of 600–700 g/cm² [22,23]. The method used to show this cell–tissue fusion (scanning electron microscopy) undoubtedly demonstrated exogenous cells fusion to the rabbit tissue, but could not show the potential occurrence of mutual cell fusion between the rabbit corneal cells. However, it is also possible that fusion did not occur between the cells of the corneal epithelial tissue for the same unknown reasons that could explain why electropermeabilized liver cells do not fuse in vivo (see discussion below).

The morphological changes observed in the B16 melanoma tissue exposed to EP are attributable to cell electrofusion, not only because of giant cells appearance (e.g., at 70 h, see Fig. 1) but also because of the extent of these changes, in the tumor volume and the kinetics of these changes.

First, the voltage initially used to treat the tumors was chosen because, in combination with bleomycin, the previously known cytotoxic effects indicated that almost all, if
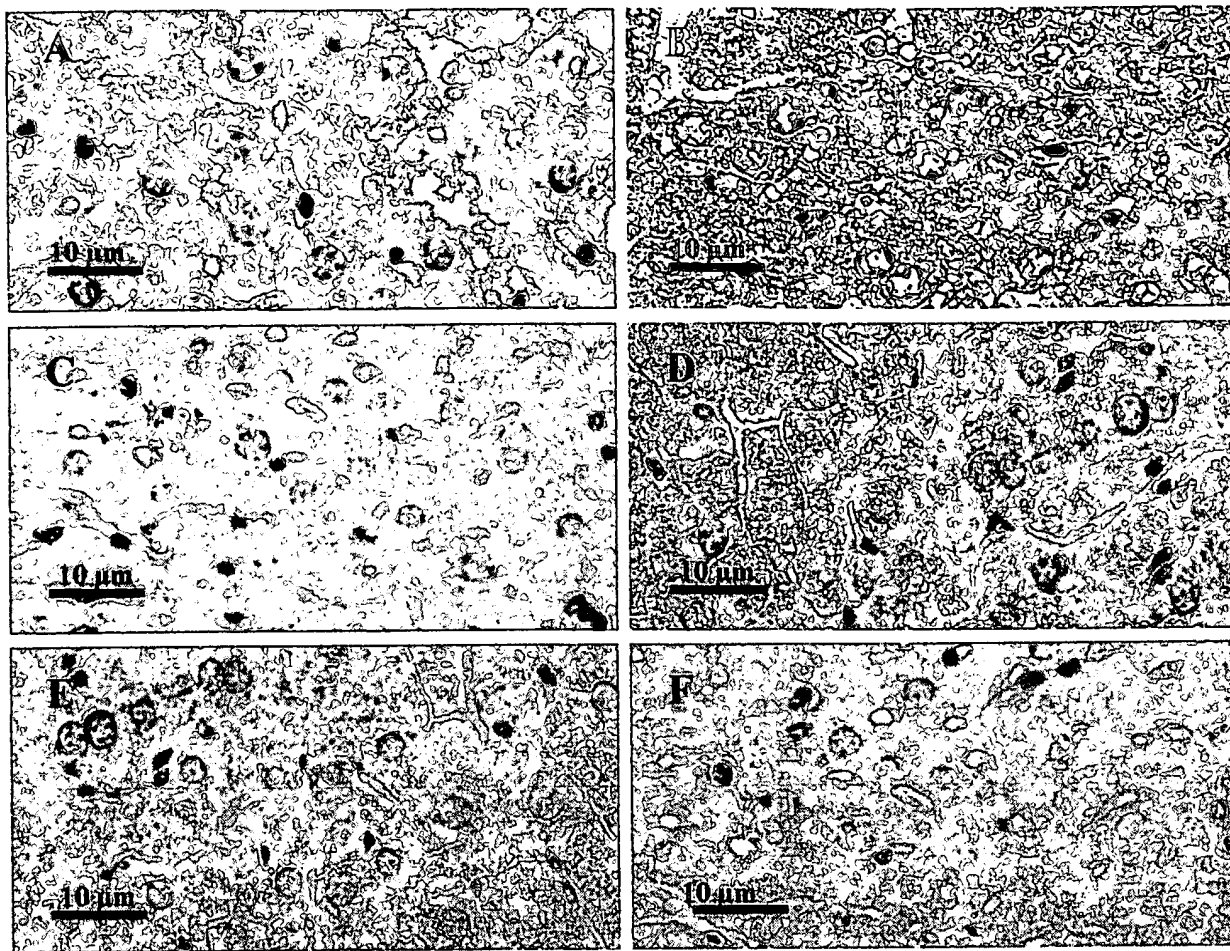
Fig. 8. Absence of electrofusion in the liver of the C57Bl/6 mice after the delivery of permeabilizing electric pulses. Liver was exposed to 8 EP of 100 μs and of 500 V/cm delivered at a frequency of 1 Hz as described in Section 2. (A) Untreated liver fixed 24 h after the treatment of the treated liver (×1300). (B) Achievement of cell electropermeabilization at 500 V/cm as revealed by the presence of pseudoapoptotic cells found, at 5 h after the electric treatment, in the liver of mice injected with 1 mg of bleomycin prior to the electric pulse delivery (×1300). (C,D) Unfused cells of livers fixed 5 h (C) or 24 h (D) after electrical treatment at 500 V/cm (×1300). (E,F) Unfused cells of livers fixed 5 h (E) or 24 h (F) after electrical treatment at 1000 V/cm (×1300).

not all the cells were permeabilized after tumor exposure to EP [35,39,40]. In parallel, fusion was detected in almost all the sections (16 out of 18 sections examined at 15 h after EP delivery). Previous experiments have also shown that the B16 tumors were only weakly affected by bleomycin when pulses of an amplitude of 500 V/cm were used. Thus we were expecting that only few cells were permeabilized at this low amplitude, instead of a large majority of the cells at 1350 V/cm. We confirmed this situation using the permeabilization test based on the effects of high doses of bleomycin on the electropermeabilized cells. In agreement with the non-homogeneity of the electric field distribution when plate electrodes are applied over a more or less spherical tumor (the highest amplitude of local electric amplitudes being found the closest to the electrodes, model not shown), permeabilization was observed only in restricted areas. In parallel, at 500 V/cm, patterns revealing cell electrofusion in B16 tumors (Fig. 6) were not detected

on the whole of the tumors sections, but only in defined regions that could correspond to the parts of the tumors which were permeabilized. Thus, in combination with the facts that (i) the number of nuclei per giant cell at 15 h using pulses of 500 V/cm was just one fourth of the number of nuclei detected using pulses of 1350 V/cm, and that (ii) syncytial areas were not detected at 500 V/cm, it can be concluded that, as expected from the theory and the in vitro results, extent of in vivo electrofusion seems to be closely related to the extent of cell electropermeabilization.

Second, it is known that, both in vitro and in vivo, permeabilization is an immediate process occurring at the time of EP delivery. It is interesting to note that in the B16 tissue exposed to the permeabilizing electric pulses the disappearance of the limits between adjacent cells was already detected at the earliest time analyzed (1 h). At that time, no change in nuclei distribution in the overall B16 tissue was detectable. Later on, nuclei distribution in the

tissue changed, separation between the cells became clear, and it was possible to detect typical giant cells similar to those obtained after cell fusion in vitro [37]. The nuclei were either all located in one group in the center of the cell, or located at the cell periphery, forming a circular pearl chain around the center of the cell. In both cases, the stain of the cytoplasm was more homogeneous and intense. The first signs of nuclei redistribution were observed at 3 h after EP delivery. Ten hours after EP, the morphological changes were completed. These morphological changes thus occurred very slowly with respect to the initial membrane perturbation, like in vitro, where it was shown that the completion of the process requires several hours and corresponds to large rearrangements of the cytoskeleton [21].

Thus, in vivo and in vitro cell electrofusion are comparable events in the sequence as well as in the kinetics of the morphological changes observed. Since it is known that electric field distribution in tissues is not homogeneous [25], the results obtained can be explained in the following way:

- at very short times after EP delivery, syncytial areas could be interpreted as the result of the disappearance of plasma membranes between the B16 cells in the tumor regions exposed to the higher local electric field values;
- in the regions exposed to intermediary local electric field values, membrane alteration was lower: fusion resulted in the formation of giant cells, a process that required important morphological changes and that could take several hours before being detectable;
- as expected, in the regions exposed to the lowest local electric field values, no fusion occurred and the B16 cells resumed their proliferation.

However, permeabilization, even if necessary, is not sufficient to obtain in vivo cell electrofusion. Indeed, tissue dependence seems to be a factor more important than cell electropermeabilization because, whatever the level of cell electropermeabilization (and thus of membrane perturbation) achieved, no electrofusion occurred in another tumor in the C57Bl/6 mice, or in a normal tissue such as liver. In the case of the LPB fibrosarcoma, even the large membrane perturbation supposed to be provoked at 2000 V/cm was unable to result in LPB cells electrofusion. Thus the observed electrofusion could be a property of the experimental melanoma used. It will be important to test the occurrence of cell electrofusion in other experimental or spontaneously arousing melanomas to understand the link of our observations either in general with the melanoma malignant transformation or in particular with the B16 melanoma. To our knowledge there is no report on fusions in mammalian tissues other than developmental fusions, such as the myoblast transforming into myotubes, or pathological fusions, like the generation of multinucleated giant cells, a characteristic feature of tuberculosis granulomas formed by the fusion of monocytes or macrophages [37].

One reasonable explanation of the differences observed between B16 and LPB tumors, as well as between B16 and normal liver, could rely on proteases release by the B16 cells:

- On the one hand, it is known that melanoma tumors are prone to produce abundant metastatic dissemination. Metastatic potential is related to proteases secretion in the environment, that facilitates the escaping of the tumor cells from the original nodule, their migration and their spreading, and that allows tumor cells invasion of new tissues through the dissociation of normal tissue structure. It is important to note that, contrary to B16 tumors, the LPB tumors do not generate metastases (unpublished data).
- On the other hand, cell treatment by trypsin (5 or 10 min at 37°C and 1 mg/ml) or pronase (5 min at 21°C or 10 min at 37°C and 20 µg/ml) resulted in a net increase of fusion yields in plated CHO cells in vitro [41]. Dispase, pronase and trypsin also facilitated the fusion of cells in suspension [42]. More recently, it has also been reported that addition of pepsin, pronase E or lysozyme leads to an increase in yields of electropermeabilization and electrofusion in U937 cells in vitro [43]. Moreover, hepatocytes isolated by collagenase treatment of the liver could be electrofused in vitro [44].

Therefore, the in vivo electrofusion of B16 cells could result from the presence of proteases in the interstitial fluid, a fact potentially linked to the B16 melanoma ability to produce metastases. The presence of proteases should reduce the extracellular matrix, the physical barrier that, by preventing the close contact of the lipid bilayers from two adjacent cells, is obviously a major obstacle to cell fusion in vivo. The absence of extracellular matrix as a factor allowing for in vitro cell–cell electrofusion was already pointed out previously in experiments in vitro [45].

The kinetics of giant cells presence in the electrically treated tissues could be explained by the occurrence of cell death and mitoses observed in these giant cells. B16 cells in control tumors not exposed to the electric pulses constitute a tissue rapidly renewing the cells that presents a level of mitoses of 4%, as well as a level of spontaneous cell death by apoptosis of 3% (H. Mekid et al., unpublished data). Fusion of cells in vivo does not seem to interfere with the entry of giant cells into mitosis. However, as one could expect, these multinucleated cells present aberrant mitotic patterns (Fig. 5). The (pseudo)-normal mitotic patterns observed in the small multinucleated cells can explain the persistence of multinucleated cells even at 100 h after EP delivery, while the decrease in the percentage of both the number of giant cells and the number of nuclei per giant cell after the peaks

observed at 15 h could result from the cell death in the large multinucleated cells illustrated by Fig. 5.

In summary, with respect to the well-established in vitro cell–cell electrofusion methodology and to the cell–tissue ex vivo and in vivo methods developed by Grasso and Heller [29–31], the new results here reported show that cell electrofusion may also occur within tissues in vivo exposed to EP. Particular microenvironmental conditions, like the existence of reduced extracellular matrices because of the presence of high levels of proteases in the interstitial medium, could be responsible for electrofusion in these tissues. Therefore, in the rapidly developing biomedical applications of in vivo electropermeabilization, some attention must be paid to the potential occurrence of cell electrofusion in the target tissue.

On the one hand, the influence of this effect on the global efficacy of DNA electrotransfer for gene therapy requires further investigation. On the other hand, the possible link between ability to metastasize and electrofusogeneity could have interesting implications in cancer treatment. Indeed, electric pulses are already used in electrochemotherapy to efficiently introduce hydrophilic cytotoxic drugs (such as bleomycin or cisplatinum) into the tumor cells. If the cells with the highest metastatic potential in the tumor cell population are fused inside the tumor, then their spreading could be reduced. Obviously, these giant cells should be subject to cell death because their electropermeabilization, which generated their fusion, should also permit cytotoxic drug uptake. However, in the case of a non-homogeneous distribution of the cytotoxic drug, or of an insufficient local supply, cell death could also occur in the cells not loaded with the drug, because, as shown here, mitoses in giant cells are altered and the death of cells in syncytial areas is triggered even in absence of cytotoxic drugs. Thus, cell electrofusion could be an important advantage of the in vivo delivery of EP to metastasizing tumors.

References

[1] W. Nickel, T. Weber, J.A. McNew, F. Parlati, T.H. Sollner, J.E. Rothman, Proc. Natl. Acad. Sci. USA 96 (1999) 12571–12576.

[2] R. Jahn, T.C. Südhof, Annu. Rev. Biochem. 68 (1999) 863–911.

[3] Z. Luo, S.R. Weiss, Virology 244 (1998) 483–494.

[4] R. Welgert, M.G. Silletta, S. Spano, G. Turaccio, C. Cericola, A. Colanzi, S. Senatore, R. Mancini, E.V. Polishchuck, M. Salmona, F. Facchiano, K.N.J. Burger, A. Mironov, A. Luini, D. Corda, Nature 402 (1999) 429–433.

[5] G.A. Neil, U. Zimmermann, Methods Enzymol. 220 (1993) 174–196.

[6] Y. Okada, Exp. Cell Res. 26 (1962) 98–107.

[7] J. Teissié, C. Ramos, Biophys. J. 74 (1998) 1889–1898.

[8] E. Neumann, S. Kakorin, K. Toensing, Bioelectrochem. Bioenerg. 48 (1999) 3–16.

[9] M.P. Rols, J. Teissié, Biophys. J. 58 (1990) 1089–1098.

[10] S. Orlowski, L.M. Mir, Biochim. Biophys. Acta 1154 (1993) 51–63.

[11] L.M. Mir, S. Orlowski, J. Belehradek Jr., J. Teissié, M.P. Rols, G. Serša, D. Miklavčič, R. Gilbert, R. Heller, Bioelectrochem. Bioenerg. 38 (1995) 203–207.

[12] U. Zimmermann, Biochim. Biophys. Acta 694 (1982) 227–277.

[13] J. Teissié, M.P. Rols, Bioelectrochem. Bioenerg. 19 (1988) 59–66.

[14] D.R. Hewish, J.A. Werkmeister, J. Immunol. Methods 120 (1989) 285–289.

[15] J. Teissié, V.P. Knutson, T.Y. Tsong, M.D. Lane, Science 216 (1982) 537–538.

[16] J. Teissié, P. Conte, Bioelectrochem. Bioenerg. 19 (1988) 49–57.

[17] C. Finaz, A. Lefevre, J. Teissié, Exp. Cell Res. 150 (1984) 477–482.

[18] P. Maurel, L. Gualandris-Parisot, J. Teissié, A.M. Duprat, Exp. Cell Res. 184 (1989) 207–218.

[19] J. Teissié, M.P. Rols, Biochem. Biophys. Res. Commun. 140 (1986) 258–266.

[20] I.G. Abidor, A.I. Barbul, D.V. Zhelev, P. Doinov, I.N. Bandrina, E.M. Osipova, S.I. Sukharev, Biochim. Biophys. Acta 1152 (1993) 207–218.

[21] C. Blangero, M.P. Rols, J. Teissié, Biochim. Biophys. Acta 981 (1989) 295–302.

[22] R.J. Grasso, R. Heller, J.C. Cooley, E.M. Heller, Biochim. Biophys. Acta 980 (1989) 9–14.

[23] R. Heller, R.J. Grasso, Biochim. Biophys. Acta 1024 (1990) 185–188.

[24] R. Heller, Anal. Biochem. 202 (1992) 286–292.

[25] J. Belehradek Jr., S. Orlowski, L.H. Ramirez, G. Pron, B. Poddevin, L.M. Mir, Biochim. Biophys. Acta 1190 (1994) 155–163.

[26] J. Gehl, T.H. Sørensen, K. Nielsen, P. Raskmark, S.L. Nielsen, T. Skovsgaard, L.M. Mir, Biochim. Biophys. Acta 1428 (1999) 233–240.

[27] J. Gehl, L.M. Mir, Biochem. Biophys. Res. Commun. 261 (1999) 377–380.

[28] P.E. Engstrom, B.R.R. Persson, L.G. Salford, Bioelectrochem. Bioenerg. 46 (1998) 241–248.

[29] L.M. Mir, F.L. Glass, G. Serša, J. Teissié, C. Domenge, D. Miklavčič, M.J. Jaroszeski, S. Orlowski, D.S. Reintgen, Z. Rudolf, M. Belehradek, R. Gilbert, M.P. Rols, J. Belehradek Jr., J.M. Bachaud, R. DeConti, B. Štabuc, P. Coninx, M. Čemažar, R. Heller, Br. J. Cancer 77 (1998) 2336–2342.

[30] L.M. Mir, S. Orlowski, Adv. Drug Deliv. Rev. 35 (1999) 107–118.

[31] M.P. Rols, C. Delteil, M. Golzio, P. Dumond, S. Cros, J. Teissié, Nat. Biotechnol. 16 (1998) 168–171.

[32] L.M. Mir, M.F. Bureau, J. Gehl, R. Rangara, D. Rouy, J.M. Caillaud, P. Delaere, D. Branellec, B. Schwartz, D. Scherman, Proc. Natl. Acad. Sci. USA 96 (1999) 4262–4267.

[33] G. Niu, R. Heller, R. Catlett-Falcone, D. Coppola, M. Jaroszeski, W. Dalton, R. Jove, H. Yu, Cancer Res. 59 (1999) 5059–5063.

[34] J. Belehradek Jr., G. Barski, M. Thonier, Int. J. Cancer 9 (1972) 461–469.

[35] L.M. Mir, S. Orlowski, J. Belehradek Jr., C. Paoletti, Eur. J. Cancer 2 (1991) 68–72.

[36] L.M. Mir, C. Roth, S. Orlowski, F. Quintin-Colonna, D. Fradelizi, J. Belehradek Jr., P. Kourilsky, J. Immunother. 17 (1995) 30–38.

[37] A. Gasser, J. Möst, Infect. Immun. 67 (1999) 395–402.

[38] L.H. Ramirez, S. Orlowski, D.J. An, G. Bindoula, R. Djodic, C. Bognel, J. Belehradek Jr., J.N. Munck, L.M. Mir, Br. J. Cancer 77 (1998) 2104–2111.

[39] G. Serša, M. Čemažar, D. Miklavčič, L.M. Mir, Bioelectrochem. Bioenerg. 35 (1994) 23–27.

[40] R. Heller, M. Jaroszeski, J. Leo-Messina, R. Perrot, N. Van Voorhis, D. Reintgen, R. Gilbert, Bioelectrochem. Bioenerg. 36 (1995) 83–88.

[41] M.P. Rols, J. Teissié, Biochemistry 29 (1990) 4561–4567.

[42] T. Ohno-Shosaku, Y. Okada, Biochem. Biophys. Res. Commun. 120 (1984) 138–143.

[43] S. Velizarov, H. Berg, Bioelectrochem. Bioenerg. 46 (1998) 263–265.

[44] J. Teissié, J.A. Reynaud, C. Nicolau, Bioelectrochem. Bioenerg. 17 (1986) 9–15.

[45] S.I. Sukharev, I.N. Bandrina, A.I. Barbul, L.I. Fedorova, I.G. Abidor, A.V. Zelenin, Biochim. Biophys. Acta 1034 (1990) 125–131.

Electrofusion of a Weakly Immunogenic Neuroblastoma with Dendritic Cells Produces a Tumor Vaccine

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The absence of surface costimulatory molecules explains in part the lack of an effective anti-tumor immune response in tumor-bearing animals, even though unique tumor antigens may be presented by class I MHC. We determined that the immunogenicity of a murine neuroblastoma, Neuro-2a, which lacks surface costimulatory molecules, could be increased by electrically induced fusion with dendritic cells. Electrofusion induced a higher level of cell fusion than polyethylene glycol, and tumor/dendritic cell heterokaryons expressed high levels of costimulatory molecules. While Neuro-2a was unable to induce the proliferation of syngeneic or allogeneic T cells *in vitro*, fused cells were able to induce T cell responses both *in vitro* and *in vivo*. When fused dendritic tumor cells were used as a cancer vaccine, immunized mice were significantly protected from challenge with Neuro-2a. We propose that electrofusion with patient-derived tumor and dendritic cells may provide a rapid means to produce patient-specific tumor vaccines. © 2001 Elsevier Science

Key Words: neuroblastoma; dendritic cells; cell fusion; cancer vaccine; cytotoxic T lymphocyte.

INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor of neonates, infants, and young children. The tumor arises from neural crest cells, which migrate to form the adrenal medulla and sympathetic ganglia during development and, therefore, can originate anywhere along the sympathetic chain (1). Neuroblastoma is characterized by aggressive local growth, followed by metastasis to regional lymph nodes, liver, bones, and the bone marrow. Patients with advanced disease (stage IV) have a 5-year survival rate of only 20–25% despite aggressive therapy (1). Prognosis in neuroblastoma depends on dissemination throughout the body. Tumors with greater dissemination usually have an amplification of the *n-myc* oncogene. An interesting exception exists in patients with stage IV-S disease. The disease in these individuals (almost all <12 months of age) is broadly disseminated but lacks amplification of the *n-myc* oncogene. Seventy-five percent of patients with IV-S disease have spontaneous remissions, possibly mediated by the immune system.

Dendritic cells (DC) are unique among antigen-presenting cells in their ability to induce antigen-specific T cell responses with extremely high efficiency (reviewed in 2, 3). Key molecular aspects which account for this ability are the high expression of both costimulatory and adhesion markers (CD80, CD86, MHC class II, ICAM-1) and T cell stimulatory cytokines (IL-1, IL-6, IL-12) (4, 5). Cognate T cell interactions further increase the ability of DC to stimulate T cell activation, as recently described for CD40–CD154 interactions (6). We sought to take advantage of these immunostimulatory functions by directly fusing dendritic cells with the weakly immunogenic murine neuroblastoma cell line, Neuro-2a. Neuro-2a was derived from a more aggressive and metastatic subclone of the C1300-NB cell line that was cultured from a spontaneous tumor in the spinal cord of a strain A/J mouse.

Dendritic cells have been "loaded" with tumor-derived material in a number of ways, each of which has induced some level of anti-tumor immunity. These strategies include incubating dendritic cells with tumor cell membranes, whole proteins, synthetic peptides derived from tumor antigens, peptides eluted from tumor cell class I MHC molecules, and even tumor cell RNA (7–11). The use of cellular fusion as a means to stimulate cellular anti-tumor immunity has been demonstrated previously by Guo *et al.* (12), who fused hepatoma cells with activated B cells, and Gong *et al.* (13), who fused dendritic cells with a carcinoma cell line. In both of these studies polyethylene glycol (PEG) was used to generate cell hybrids. Our report builds upon these studies in two ways: first, by the use of dendritic cells instead of B cell blasts, and secondly, by the immediate use of the fused cellular vaccines (as

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opposed to continued *in vitro* culture). While tumors of a similar tissue origin may or may not express conserved tumor-specific antigens, each tumor certainly has randomly accumulated genetic lesions, making the individual tumor itself an attractive source of material for inducing a specific anti-tumor immune response (14). This heterogeneity of tumor antigens may explain why therapy based on single antigens does not always work (15, 16). If these principles hold for neuroblastoma, then fusing a patient's own tumor cells with autologous dendritic cells may prove to be an ideal cancer vaccine.

MATERIALS AND METHODS

Cell lines and generation of DC. The Neuro-2a cell line was obtained from the ATCC (Manassas, VA) and expanded in culture, and "master" cell lines were frozen. All live tumor-challenge experiments were carried out with the same frozen master cell line. Neuro-2a cells were thawed and cultured less than 1 week prior to use in tumor-challenge experiments. DC were generated from bone marrow collected from A/J (H-2^a) or C57BL/6 (H-2^b) mice (The Jackson Laboratory, Bar Harbor, ME). Bone marrow mononuclear cells were isolated after separation on Ficoll/Hypaque and cultured in 6-well plates (3-ml vol) in DMEM supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-gln, 0.136 μM folic acid, 27.2 mM asparagine, 0.137 M l-arginine, penicillin–streptomycin, 5 $\times$ 10^{-5} β-mercaptoethanol (cDMEM, all media components from Life Technologies, Gaithersburg, MD), 10 ng/ml GM-CSF, and 100 U/ml IL-4 (cytokines from ID Labs Biotechnology, London, ON, Canada). On days 2 and 4, the culture was fed by exchanging half of the medium with fresh cDMEM supplemented with GM-CSF and IL-4. On day 5, the nonadherent cells were transferred to a 24-well plate at 2 $\times$ 10^6 cells per well in fresh medium with cytokines, and the DC were "differentiated" with 20 μg/ml poly(I)–poly(C) (Sigma Chemical Co., St. Louis, MO; P0913). On day 7 of culture, the DC were harvested. DC populations generated were greater than 99% positive for CD11c, DEC205, and H-2K^k as determined by flow cytometry.

Fluorescent labeling of cells. To detect fused cells and quantify fusion, cells were stained with CMFDA (5-chloromethylfluorescein diacetate) or CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl)amino)tetramethylrhodamine) (Molecular Probes, Inc., Eugene, OR), as described by Jaroszeski *et al.* (17). Briefly, 5 mM stock solutions of each were prepared in DMSO, cells were brought to 1.5 $\times$ 10^6/ml in DMEM, and either 1 μl CMFDA or 9 μl CMTMR per 20 ml of cell suspension was added. Cells were incubated at 37°C for 30 min in the dark, washed once with DMEM/10% FBS, resuspended in the original volume, and incubated another 40 min at 37°C in the dark. The stained cells were washed twice and prepared for fusion.

Cell Fusion

PEG fusion. A total of 1 $\times$ 10^6 tumor cells and DC, at different ratios, were combined and washed twice with serum-free DMEM. All traces of wash media were removed, and the cells were resuspended in 0.5 ml 50% PEG 1450 (ATCC) at 37°C in PBS, pH 7.5, over 1 min while the tube was gently mixed. This was followed by the addition of 0.5 ml prewarmed PBS over 1 min with gentle mixing, another 0.5 ml over 1 min, and then an additional 10 ml of PBS over 3 min. The tube was allowed to stand at room temperature for 10 min, and then the cells were pelleted by centrifugation, resuspended in 2 ml of IMDM (Life Technologies, phenol red-free) supplemented with 20% FBS (Life Technologies), and incubated in a tissue culture incubator in 24-well plates. Unfused controls were similarly treated, omitting PEG exposure. Cells were rested 1 h prior to flow cytometric analysis.

Electrofusion. Dye-loaded tumor cells were washed 1$\times$ in PBS and then exposed to trypsin/PBS (Life Technologies) for 5 min at 37°C. Trypsinization was stopped by the addition of 10% FBS, and the cells were pelleted and then washed three times in freshly prepared glucose fusion media (GFM, 0.3 M d-glucose, 18 mM MgCl$_2$, 18 mM CaCl$_2$, 1 mM HEPES, pH 7.2). DC were washed separately in GFM. Washed DC were resuspended in 80 μl GFM and added to pelleted tumor cells. The cellular mixture was transferred to a BT450 microscope slide fusion chamber (0.5-mm gap divergent field), 25 μl per fusion. The cells were fused using an ECM 2001 apparatus (Genetronics, Inc., BTX Instruments, San Diego, CA). Cells were aligned for 19 s with 25 V AC, pulsed with three consecutive 30-μs pulses of 80 V DC, then reexposed to 25 V AC for 9 s to promote postfusion adherence. After exposure to electric current, the cells were immediately flushed from the fusion slide into a 24-well plate and cultured for 1 h in IMDM plus 20% FBS. For larger cell numbers the BT453 (3.2-mm gap, homogeneous field; 0.5-ml vol) fusion slide was used. Conditions for this chamber were DC voltage, 600 V for three 30-μs pulses, prefusion AC alignment, 35 V for 22 s, postfusion AC alignment for 9 s at 35 V.

Cell surface markers. Expression of extracellular proteins was examined by three-color flow cytometry using a Becton-Dickinson (San Jose, CA) FACScan and FlowJo software (Tree Star, Inc., San Carlos, CA) for analysis. DC and Neuro-2a cells were first stained with CMTMR or CMFDA, respectively. Cells were incubated with the following reagents: biotinylated antibodies specific for I-A^d, H-2K^d, CD80, CD86, CD54 (PharMingen, San Diego, CA), or a combination of

DEC205 (Serotec, Kidlington, Oxon, UK; supplied as culture supernatant) and biotinylated anti-rat IgG2a. Following incubation with the biotinylated reagents, cells were stained with streptavidin–CyChrome (PharMingen). For *in vivo* depletion studies, CD4 cells were depleted with anti-L3T4 (clone GK1.5; ATCC), CD8 cells with anti-Lyt2.2 (clone 2.43; ATCC), and NK cells with anti-asialo-GM1 (Wako Pure Chemical Industries Ltd., Osaka, Japan).

T cell proliferation assays. Neuro-2a cells (A/J origin) were treated with 8000 rad, mixed with C57BL/6 DC, and electrofused or not fused. The fused and non-fused cells were tested for the ability to induce proliferation of C57BL/6 T cells *in vitro*. The T cells were purified by positive selection using a MACS magnetic cell separator (Miltenyi Biotec) after incubation with anti-Thy1.2-conjugated microbeads. Briefly, various concentrations of the fused and nonfused cells were plated in triplicate U-bottom microwells with 10^5 C57BL/6 T cells. After 4 days of culture, 1 μCi of [^{3}H]thymidine was added to each well. Following an additional 24 h of culture, the cells were harvested and assessed for proliferation as determined by [^{3}H]thymidine uptake. Delta cpm values were calculated as the average cpm of T cells cultured with fused or nonfused cells minus the average cpm of T cells cultured with DC alone.

Immunization and tumor challenge. A/J mice were immunized with two weekly intraperitoneal (ip) or subcutaneous (sc) injections of 0.5–1 × 10^6 irradiated (3000 rad) Neuro-2a tumor cells, tumor mixed with DC, or tumor cells electrofused with DC. Seven days after the second immunization, the mice were challenged with 10^6 Neuro-2a cells. Mice immunized ip or sc were challenged at the same site. Animals were monitored daily for evidence of tumor and sacrificed when tumors prevented feeding or ambulation or if the tumor ulcerated through the skin. Animals were housed at the Medical College of Wisconsin Animal Resource Center, an AAALAC-certified facility.

CTL assay. Spleens were collected from control or immunized animals 1 week following the second immunization and processed into single cell suspension, and the T cells were isolated by positive selection using a MACS magnetic cell separator after incubation with anti-CD4 and anti-CD8-conjugated beads. Purified T cells were cultured for 1 week with mitomycin-C-treated Neuro-2a cells and IL-2. On day 7 of culture, stimulated T cells were assayed for lytic activity versus ^{51}Cr-labeled Neuro-2a. Six wells were set up with targets alone and targets plus detergent to determine spontaneous and maximum release values, respectively. The percentage of specific lysis was calculated by the following formula:

TABLE 1
Comparison of Electrofusion to PEG-Mediated Fusion

Ratio of DC to tumor[b]	% Total cells fused[a]	
	PEG-mediated	Electrofusion
20:80	2.0	5.9
30:70	1.4	10.2
40:60	1.9	6.9
50:50	1.3	5.1
60:40	1.9	10.2
70:30	2.3	3.1
80:20	2.2	5.1

Note. Fusion of dendritic cells to tumor cells was determined by loading cells with either CMFDA or CMTMR, carrying out PEG-mediated cell fusion or electrofusion (as described under Materials and Methods), and then analyzing the fused population of cells by flow cytometry. Cells that gave a positive fluorescence signal for both CMFDA and CMTMR were considered fused. This experiment is representative of three separate determinations which gave similar results.

[a] Dye-loaded DC and tumor cells were mixed at various ratios. The cells were then "fused" or not fused (see Materials and Methods for details) and analyzed by flow cytometry to determine the percentage of total cells fused. Percentage fusion was determined by subtracting the percentage of double-positive cells of the unfused cells (i.e., not exposed to PEG or electric current) from the double-positive cells that underwent the respective fusion procedure.

[b] A total of 1 × 10^6 cells was used for PEG-mediated fusion experiments while a total of 5 × 10^5 cells was used for each electrofusion.

% specific lysis (chromium release)

$$= \frac{\text{cpm experimental} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100.$$

Statistics. Survival curves were compared using the log-rank test. Percentage fusion values were compared using the Student t test.

RESULTS

The ability to fuse DC and tumor cells was evaluated using two different methods, PEG-mediated cell fusion and electrofusion. To determine the fusion rates, DC and Neuro-2a tumor cells were loaded with either a fluorescein (CMFDA)- or rhodamine (CMTMR)-based intracellular dye (see Materials and Methods) mixed at various ratios and fused (Table 1). After completion of the fusion process, the cells were analyzed by two-color flow cytometry and the percentage fusion was calculated. Since CMFDA and CMTMR are intracellular dyes, the binding of membrane fragments from cells that may have lysed during the fusion process to the surface of the viable gated cells was not a concern, as would have been if lipid-soluble dyes were used. Fusion was scored as the percentage of nonfused dual-positive

cells (i.e., mixtures of DC and tumor cells not exposed to direct current voltage or PEG) subtracted from the percentage of dual-positive cells exposed to electrical current or PEG. This was to control for any spontaneous cell-to-cell adhesion that occurred during the electrofusion and PEG fusion procedures. The nonfused controls were typically less than 2% dual-positive (data not shown). The data in Table 1 demonstrate that electrofusion was clearly superior to PEG-mediated fusion at all DC:tumor ratios tested. Neither of the DC: tumor cell ratios was vastly superior in terms of percentage tumor cells fused. To directly compare electrofusion and PEG-mediated cell fusion, DC and Neuro-2a cells were labeled with CMTMR and CMFDA, respectively, mixed at a 60:40 ratio, and then fused with electrical current or PEG (as described under Materials and Methods). Unfused cell mixtures were included as controls. Electrofusion resulted in a significantly higher percentage of fused cells than PEG-mediated fusion, giving an average fusion rate of 12.4 ± 5.9 percent compared to an average fusion rate of 1.8 ± 0.8 percent for PEG ($P = 0.037$, $n = 5$). Electrofusion achieved fusion rates of 8–21 percent, while PEG never induced more than 3 percent fusion. These data demonstrate that electrofusion was superior to PEG-mediated fusion.

To determine whether electrofusion of DC to tumor cells altered the expression of cell surface antigens important for antigen presentation, three-color flow cytometric analysis was performed after electrofusion of CMFDA-labeled Neuro-2a cells to CMTMR-labeled DC. Cell surface markers were detected by incubating cells with specific biotin-conjugated antibodies followed by streptavidin-cy-chrome. As demonstrated in Fig. 1A, fused DC–tumor cells could be clearly distinguished as a dual-stained population of cells. When the fused cells were gated and analyzed for various cell surface markers using a third fluorescent marker, the fused DC–tumor cells maintained or even increased expression of CD80 (B7-1), CD86 (B7-2), and CD54 (ICAM-1) (Fig. 1B). DC-specific class I and class II MHC molecules, as well as the DEC205 antigen found on DC, were also expressed by the fused cells (Fig. 1B and data not shown).

It is known that DC can take up apoptotic bodies from tumor cells in tumor–dendritic cell coincubation experiments (18). Although the time between electrofusion and flow cytometric analysis is less than 1 h, we wanted to insure that the uptake of apoptotic bodies or exosomes was not responsible for dual-fluoresence and thus counted as a false-positive fusion rate. We either performed our standard fusion procedure (2 × 10⁶ cells total, 60:40 ratio of A/J-derived DC to Neuro2a), or omitted either the DC or the tumor cells from the fusion reaction, subsequently coincubated the cell population exposed to electric current with its fusion partner for 90 min (at least twice the time period used

under Materials and Methods), and analyzed the coincubated cell populations by flow cytometry. When CM-FDA-labeled Neuro-2a were fused to CMTMR-labeled DC, a fusion rate of 21% was recorded. When these cell populations were coincubated for 90 min without exposure to current, a background fusion rate of 3% was seen. When tumor cells alone were exposed to current and then mixed with nonexposed DC, a 2% fusion rate was recorded. Exposing tumor to the electrical conditions used to induce fusion, therefore, did not increase the fluorescence signal. When DC and Neuro-2a were exposed to electrical current in separate fusion reactions and then coincubated, a background fusion rate of 3% again was seen (these data are representative of three separate experiments). Thus, under the conditions used to analyze fusion, dual-positive fluorescence is not increased by exposure to electric current.

Next, we examined whether fused DC–tumor cells could stimulate a T cell response *in vitro*. Irradiated Neuro-2a cells (MHC class I⁺,II⁻) were mixed with allogeneic MHC-disparate (C57BL/6) DC at a 40:60 ratio and electrofused or not fused. The fused and nonfused cells were then tested for their ability to stimulate the proliferation of purified C57BL/6 T cells (syngeneic to the DC) *in vitro*. Thus, any proliferation that occurred would be due to recognition of Neuro-2a-derived class I MHC–peptide complexes on the fused cells. Neuro-2a cells alone were incapable of stimulating the proliferation of allogeneic C57BL/6 T cells (data not shown). Likewise, nonfused mixtures of Neuro-2a and C57BL/6 DC failed to induce the proliferation of C57BL/6 T cells higher than that observed when T cells and DC were cocultured alone ("autologous" stimulation) (Fig. 2). In contrast, fused Neuro-2a–C57BL/6 DC induced a significant T cell response *in vitro*, indicating that Neuro-2a-derived cell surface antigens on the fused cells could now be recognized by the C57BL/6 T cells.

The high level of costimulatory molecule expression on fused dendritic cell–tumor cell hybrids, and their ability to stimulate the proliferation of T cells *in vitro*, led us to evaluate their ability to serve as a cellular vaccine for neuroblastoma. DC were cultured from bone marrow, electrofused to irradiated Neuro-2a cells, and injected either ip or sc. One week after the second of two weekly vaccinations, the immunized mice were challenged with viable Neuro-2a via the same route as vaccination. As shown in Fig. 3A, ip immunization with Neuro-2a cells alone provided no protective benefit from tumor challenge compared to nonimmunized mice (20 vs 27% survival, respectively). In contrast, mice immunized ip with fused cells had significantly better survival (80%) than the nonimmunized mice (27% survival), mice immunized with tumor only (20% survival), or the mice immunized with nonfused cells (40% survival) ($P < 0.017$). The mice immunized with nonfused cells survived longer and better than the nonim-
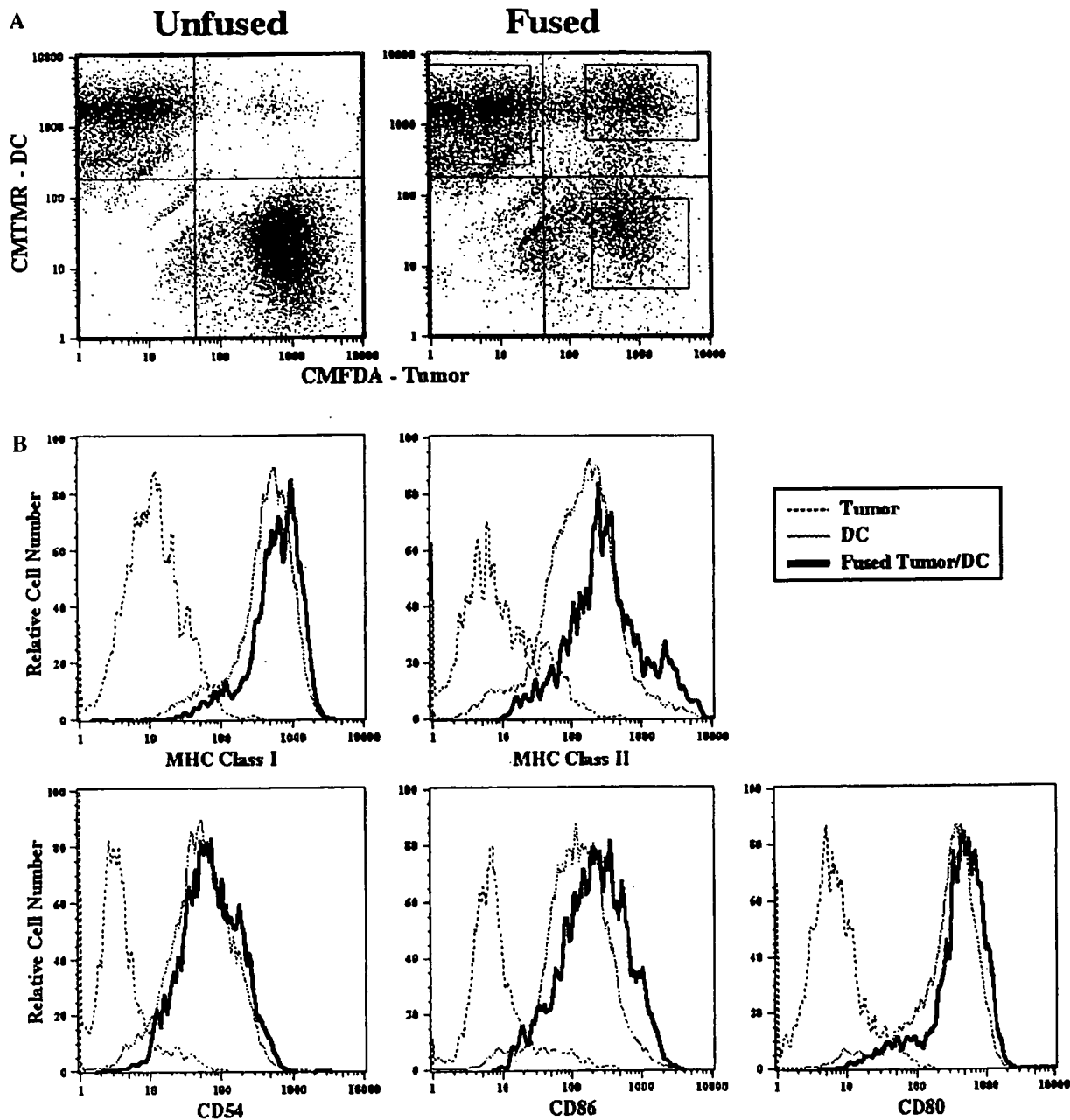
FIG. 1. Expression of costimulatory cell surface proteins on fused DC–tumor cells. CMTMR-loaded dendritic cells and CMFDA-loaded Neuro-2a (tumor) were electrofused and analyzed by two-color flow cytometry (see Materials and Methods for details). (A) Dot-plots demonstrate a clear fused cell population that could be readily selected for analysis of cell surface markers using the cy-chrome fluor. (B) Three-color flow cytometric analysis of a fused DC–tumor cell population stained for expression of CD54, CD80, CD86, and class and class II MHC (see Materials and Methods). Cell surface expression of the three gated populations is plotted as solid line, fluorescence of the fused population (upper right quadrant in A); thin gray line, analysis of DC alone (upper left quadrant in A); dashed line, signal for unfused Neuro-2a alone (lower right quadrant in A).

munized or tumor-immunized mice, but the differences were not statistically significant. These results indicated that ip immunization with fused Neuro-2a–DC induced a protective immune response *in vivo*.

When mice were immunized sc (Fig. 3B), the differential protective effect of fused dendritic cell–tumor cell hybrids was not as profound, most likely due to the

fact that immunization with irradiated tumor alone induced a partially protective immune response compared to the nonimmunized mice (33 vs 0% survival: $P = 0.013$). Nevertheless, sc immunization with fused cells provided a significantly better protective effect than tumor-immunization (80 vs 33% survival: $P = 0.009$). The fused-cell-immunized mice had a higher
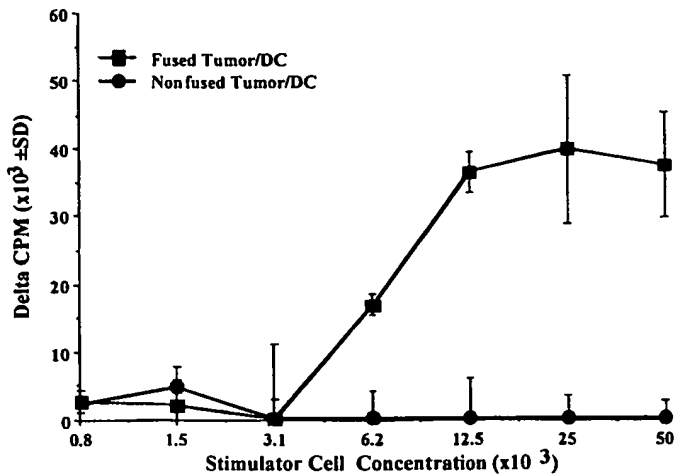
FIG. 2. Neuro-2a cells fused with allogeneic DC induced a T cell response *in vitro*. Neuro-2a tumor cells (A/J origin) were treated with 8000 rad, mixed with C57BL/6 DC, and electrofused or not exposed to current as a control. Fused and nonfused Neuro-2a/DC cells were tested for the capacity to induce proliferation of C57BL/6 purified T cells *in vitro* (see Materials and Methods for details). Delta cpm values were calculated as the average cpm of C57BL/6 T cells cultured with fused or nonfused stimulators (Neuro-2a + C57BL/6 DC) minus the average cpm of T cells cultured with DC alone.

survival rate than the nonfused-cell-immunized mice (80 vs 60%), but the difference in survival was not statistically significant.

To determine whether immunization with fused DC–tumor cells induced a CTL response *in vivo*. mice were sacrificed from each group of ip-immunized mice (fused, nonfused, tumor only) on the day of tumor challenge. Normal nonimmunized control mice were also sacrificed. The spleens were harvested and processed into single cell suspension, and T cells were isolated with a MACS magnetic cell separator. T cells were cultured for 1 week with irradiated Neuro-2a cells and IL-2. Seven days later, the cells were tested for cytolytic activity against ^{51}Cr-labeled Neuro-2a target cells (Fig. 4). The T cells isolated from nonimmunized mice and mice immunized with tumor only did not develop cytolytic activity. Interestingly, mice immunized with either fused tumor–DC or unfused cell mixtures both exhibited cytolytic activity versus Neuro-2a.

To further investigate the participation of different immune cell subsets in the generation of protective immunity using an electrofused tumor cell–DC vaccine, we examined the effect of depleting CD4, CD8, or NK cells using anti-CD4, anti-CD8, or anti-asialo-GM1 antibody in our tumor vaccine model (Fig. 5). Strain A/J mice were immunized ip twice weekly with fused Neuro-2a–DC and then challenged 1 week later with 1×10^6 viable Neuro-2a tumor cells. In our desire to maximize vaccine efficacy, all cells from each fusion procedure were utilized for immunization. Fusion rates ranged from 5.3 to 14% (average = 9.7 ± 3.2), and the total number of cells injected ranged from 3.7×10^5 to

7.2×10^5 (average = $5.6 \times 10^5 \pm 1.4 \times 10^5$). As anticipated, fused cells provided these mice a significant survival advantage over nonimmunized mice. Survival rates were 80% for mice immunized with DC–Neuro-2a fusions as opposed to 18% for nonimmunized mice ($P = 0.0048$). When mice were immunized with fused DC–Neuro-2a fusion and depleted of CD8 cells, the survival rate fell to 10%, $P = 0.00087$, compared to nonantibody-treated mice. Immunized mice treated with anti-CD4 antibody did not have a significant impairment in survival. When immunized mice were treated with anti-asialo-GM1 antibody in order to deplete NK cells, the survival rate decreased to approximately 35%, $P = 0.0568$, in comparison to nonantibody-treated immunized animals. Thus, both NK and CD8 cells appear to play a role in the protective immune response to Neuro-2a challenge.

DISCUSSION

The fusion of dendritic cells to tumor cells has rapidly become the focus of a number of clinical trials. Recent results in a trial with metastatic renal cell carcinoma patients demonstrated the partial efficacy and safety of DC–tumor cell hybrid vaccines (19). In this clinical protocol a number of patients showed either cure or marked reduction in tumor burden. In our studies with a murine model of neuroblastoma, we found that electrofusion was superior to the PEG-mediated fusion, with the clinical implication that an even more potent vaccine could be produced using this methodology. Preliminary studies in our laboratory have demonstrated the ability to electrofuse human dendritic cells with neuroblastoma cell lines as well. Gong *et al.* cultured PEG-generated DC–tumor cell hybrids for up to 1 week in selective growth media (20). Our goal was to establish a procedure wherein fused DC–tumor cells could be immediately given as a tumor vaccine. We found that electrofusion generated herterokaryons in a rapid manner that could potentially be used with primary tumor tissue.

The availability of an animal model to study neuroblastoma has opened several new lines of investigation. The murine and human diseases are similar in pathology and, more importantly, in the demonstration of an interaction between the immune system and the tumor. Both human and murine neuroblastomas have low levels of MHC class I expression, which can be upregulated by treatment with cytokines, and both are capable of eliciting some level of cellular and humoral immune reactivity (21). Recent approaches to treating neuroblastoma in murine systems have incorporated advances in molecular biology to "engineer" the tumor so that it induces a more efficient cellular immune response. Some of the most impressive experimental anti-tumor effects were obtained when neuroblastoma cells were cotransfected with granulocyte/monocyte-
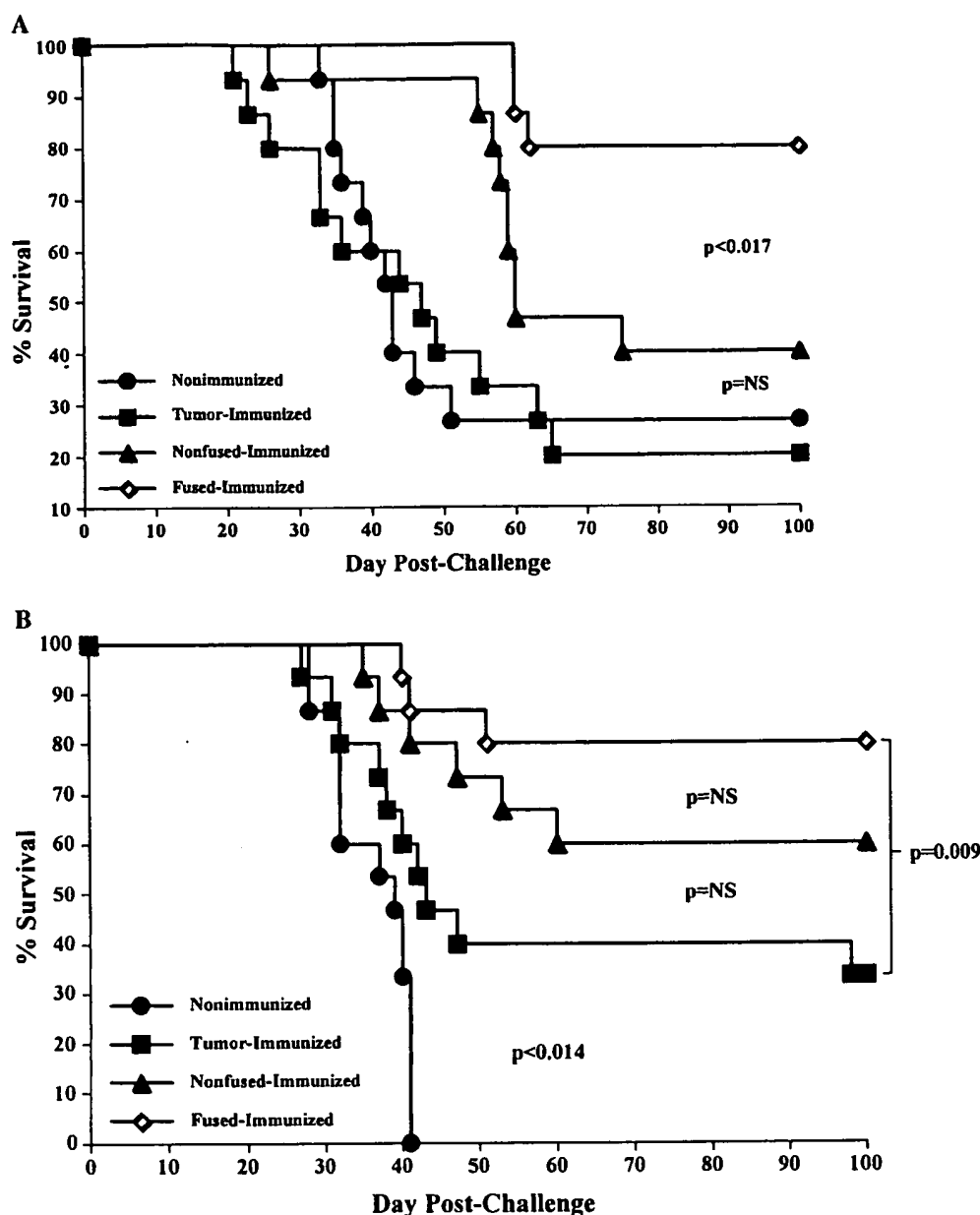
FIG. 3. Immunization with fused DC–Neuro-2a cells induced a protective immune response *in vivo*. A/J mice were immunized with two weekly (A) intraperitoneal or (B) subcutaneous injections of irradiated (3000 rad) Neuro-2a tumor cells, tumor mixed with A/J DC, or electrofused tumor–DC. The cellular dose ranged from 0.5- to 1×10^6, depending on the final yield of cells from the fusion procedure. Identical numbers of viable tumor, tumor mixed with DC, or tumor fused with DC were infused for each vaccination. Seven days after the second immunization, the mice were challenged via the same site. Each experimental group consisted of 15 mice. The data represent the combined results of three experiments.

colony stimulating factor (GM-CSF) and interferon-γ (22). Histological examination of tumor lesions in vaccinated mice demonstrated an infiltration of dendritic cells, supporting the hypothesis that transfected tumor cells do not present antigen directly. Rather, antigen is taken up by antigen-presenting cells, which then migrate to regional lymph nodes, where specific T cell responses are induced (23, 24). These studies add support for a dendritic cell-based tumor vaccine in the treatment of neuroblastoma. Furthermore, the ability to alter the immunogenicity of the tumor without requiring transfection with recombinant DNA or transduction with viral gene vectors allows for rapid translation to the clinic. To that end, we investigated whether dendritic cells directly fused to Neuro-2a could transfer their immunostimulatory capacity and induce a protective anti-tumor immune response *in vivo*. Electrofusion did not diminish the expression of
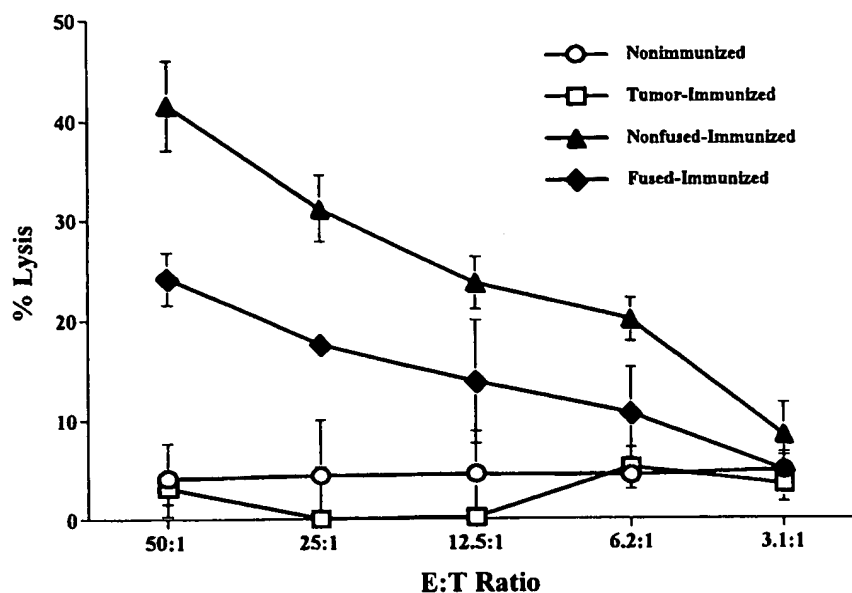
FIG. 4. Generation of CTL from immunized animals. A/J mice were immunized with irradiated Neuro-2a tumor cells (tumor-immunized), nonfused mixtures of Neuro-2a and DC (nonfused-immunized), or fused Neuro-2a/DC (fused-immunized) or were not immunized. T cells isolated from immunized mice were cultured for 1 week with irradiated Neuro-2a cells and rIL-2. Following culture, the lytic activity of tumor-reactive lymphocytes was tested in a standard chromium-release assay using Neuro-2a cells as targets.

dendritic cell-specific costimulatory molecules, demonstrating that cell surface markers known to break tolerance and induce immune responses to tumors were now directly transferred to the tumor by membrane fusion. Electrofusion also preserved the expression of DC-derived class I and class II MHC molecules on the
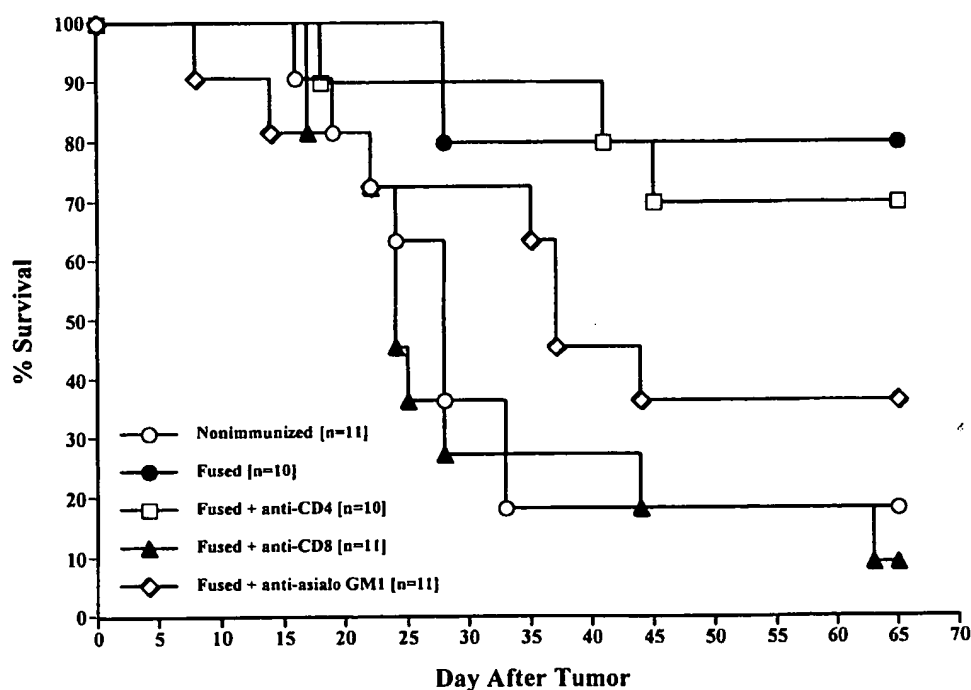

FIG. 5. Participation of CD8⁺ and NK cells in vaccine-induced immunity to Neuro-2a. Mice were either not immunized or immunized and selectively depleted of immune cell subsets with antibody. Immunized mice were injected ip twice weekly with fused DC-Neuro-2a (60:40 ratio of DC to tumor, cell dose ranging from 3.7×10^5 to 7.2×10^5). One week later mice were challenged ip with 1×10^6 Neuro-2a and monitored for tumor progression. For antibody depletion, a total of six injections was carried out. Respective mice were antibody-depleted with 250 μg of antibody (for CD4 and CD8, or 50 μl of anti-asialo-GM1) the day of and 2 days after each immunization and on the day of and 2 days after the challenge date. Immunized mice were depleted of CD4⁺ cells, CD8⁺ cells, or NK cells.

surface of the fused cell. In future studies, we will examine the ability of these transferred MHC molecules to present neuroblastoma-derived antigens.

Neuro-2a on its own showed a differential ability to induce anti-tumor immunity when animals were immunized ip versus sc. Animals vaccinated ip were not protected from tumor challenge after immunization with tumor alone, while some animals vaccinated sc with tumor alone survived tumor challenge (Fig. 3). Our data are thus in agreement with other studies demonstrating the efficacy of sc injections which initiate T cell immune responses in the local draining lymph nodes (25). Perhaps antigens from dying tumor cells are taken up by endogenous DC at the site of sc inoculation, resulting in a relatively modest local immune response followed by a modest systemic response.

It is clear from the data in Fig. 3 that fusion of Neuro-2a with DC produced the most effective vaccine. The tumor-protective immune response induced by fused DC–tumor cells correlated with the ability to generate tumor-reactive CTL from the spleens of immunized animals (Fig. 4). However, the fact that CTL could also be generated from the spleens of mice immunized with unfused mixtures of DC and tumor cells suggests that CTL activity may not be the best indicator of protective immunity. Suboptimal immunity may be induced by unfused DC that take up tumor antigens at the site of injection. Future studies are planned to assess the immune response at more localized sites (i.e., regional lymph nodes and the tumor lesion itself) as well as in the spleen.

To examine our observation that more than one mechanism of anti-tumor immunity might be induced by our fusion vaccine, we carried out antibody depletion studies *in vivo* to determine the relative importance of CD4, CD8, and NK cell subsets (Fig. 5). These data demonstrate that while anti-CD4 antibody did not abrogate tumor immunity, both anti-CD8 and anti-NK antibody did. Even though the depletion of CD8 cells had a more dramatic effect, it is clear that NK cells play a role in the tumor immunity induced by electrofused DC–tumor preparations. Preliminary experiments examining the sensitivity of Neuro-2a to either NK or LAK cells indicate that Neuro-2a is NK-cell-insensitive but LAK-sensitive (not shown). This may indicate that NK cells are an integral part of the inflammatory process, perhaps through the release of cytokines that amplify tumor immunity in tumor sites, but not the ultimate effector cells in anti-Neuro-2a immunity.

The fusion of dendritic cells to tumor cells by exposure to an electric field in a device especially designed to induce cell-to-cell contact via AC current is superior to PEG-mediated fusion and can be rapidly expanded to include other tumor cell types. Fused tumor–DC cells express T cell costimulatory and MHC molecules

on their surface and induce tumor immunity *in vivo*. We propose that the unique combination of MHC molecules from the tumor (bearing tumor-derived peptides) and the costimulatory molecules derived from the DC induces anti-tumor immunity in this vaccine setting. Future studies will examine the relative roles of antigen presentation by the DC–tumor cell heterokaryons and bone marrow-derived antigen-presenting cells in our tumor challenge model, as well as to examine the efficacy of DC–tumor fusions in a treatment model for neuroblastoma.

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REFERENCES

1. Isaacs, H., Jr., Neuroblastoma. *In* "Tumors of the Fetus and Newborn," pp. 130–149, Saunders, Philadelphia. 1997.

2. Steinman, R. M., The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271–296, 1991.

3. Hart, D. N. J., Dendritic cells: Unique leukocyte populations which control the primary immune response. *Blood* **90**, 3245–3287, 1997.

4. Kanangat, S., Nair, S., Babu, J. S., and Rouse, B. T., Expression of cytokine mRNA in murine splenic dendritic cells and better induction of T cell-derived cytokines by dendritic cells than by macrophages during *in vitro* costimulation assay using specific antigens. *J. Leukocyte Biol.* **57**, 310–316, 1995.

5. Larsen, C. P., Ritchie, S. C., Pearson, T. C., Linsley, P. S., and Lowry, R. P., Functional expression of the costimulatory molecule B7/BB1 on murine dendritic cell populations. *J. Exp. Med.* **176**, 1215–1220, 1992.

6. Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Kooten, C. V., Durand, I., and Banchereau, J., Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* **180**, 1263–1272, 1994.

7. Young, J. W., and Inaba, K., Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J. Exp. Med.* **183**, 7–11, 1998.

8. Mayordomo, J. I., Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Melief, C. J., Ilstad, S. T., Kast, W. M., and Lotze, M. T., Bone marrow-derived dendritic cells pulsed with synthetic tumor peptides elicit protective and therapeutic antitumor immunity. *Nat. Med.* **1**, 1297–1302, 1995.

9. Bakker, A. B. H., Marland, G., de Boer, A. J., Huijbens, J. F., Danen, E. H. J., Adema, G. J., and Figdor, C. G., Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells *in vitro*. *Cancer Res.* **55**, 5330–5334, 1995.

10. Flamand, V., Sornasse, T., Thielemnans, K., Demanet, C., Bakkus, M., Bazin, H., Tielemans, F., Leo, O., Urbain, J., and Moser, M., Murine dendritic cells pulsed *in vitro* with tumor antigen induce tumor resistance *in vivo*. *Eur. J. Immunol.* **24**, 605–610, 1994.

11. Mannering, S. I., McKenzie, J. L., Fearnley, D. B., and Hart, D. N. J., HLA-DR1-restricted bcr-abl (b3a2)-specific CD4$^+$ T lymphocytes respond to dendritic cells exposed to b3a2 containing cell lysates. *Blood* **90**, 290–297, 1997.

12. Guo, Y., Wu, M., Chen, H., Wang, X., Liu, G., Li, G., Ma, J., and Sy, M.-S., Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells. *Science* **263**, 518–520, 1994.

13. Gong, J., Chen, D., Kashiwaba, M., and Kufe, D., Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat. Med.* **3**, 558–561, 1997.

14. Tamura, Y., Peng, P., Liu, K., Daou, M., and Srivastava, P. K., Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* **278**, 117–120, 1997.

15. Ramarathinam, L., Sarma, S., Maric, M., Zhao, M., Yang, G., Chen, L., and Liu, Y., Multiple lineages of tumors express a common tumor antigen, P1A, but they are not cross-protected. *J. Immunol.* **155**, 5323–5329, 1995.

16. Speiser, D. E., Miranda, R., Zakarian, A., Bachmann, M. F., McKall-Faienza, K., Odermatt, B., Hanahan, D., Zinkernagel, R. M., and Ohashi, P. S., Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: Implications for immunotherapy. *J. Exp. Med.* **186**, 645–653, 1997.

17. Jaroszeski, M. J., Gilbert, R., and Heller, R., Detection and quantitation of cell–cell electrofusion products by flow cytometry. *Anal. Biochem.* **216**, 271–275, 1994.

18. Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S., Eradication of established murine tumors using a novel cell-free vaccine: Dendritic cell-derived exosomes. *Nat. Med.* **4**, 594–600, 1998.

19. Kugler, A., Stuhler, G., Walden, P., Zöller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Müller, C. A., Becker, V.,

Gross, A. J., Hemmerlein, B., Kanz, L., Müller, G. A., and Ringert, R.-H., Regression of human metastatic renal cell carcinoma after vaccination with tumor cell–dendritic cell hybrids. *Nat. Med.* **6**, 332–336, 2000.

20. Gong, J., Avigan, D., Chen, D., Wu, Z., Koido, S., Kashiwaba, M., and Kufe, D., Activation of antitumor cytotoxic T lymphocytes by fusions of human dendritic cells and breast carcinoma cells. *Proc. Natl. Acad. Sci. USA* **97**, 2715–2718, 2000.

21. Katsanis, E., Blazar, B. R., Bausero, M. A., Gunther, R., and Anderson, P. M., Retropertitoneal inoculation of murine neuroblastoma results in a reliable model for evaluation of the antitumor immune response. *J. Pediatr. Surg.* **29**, 538–542, 1994.

22. Bausero, M. A., Panoskaltsis-Mortari, A., Blazar, B. R., and Katsanis, E., Effective immunization against neuroblastoma using double-transduced tumor cells secreting GM-CSF and interferon-γ. *J. Immunother.* **19**, 113–124, 1996.

23. Huang, A. Y. C., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D. M., and Levitsky, H., Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* **264**, 961–965, 1994.

24. Bannerji, R., Arroyo, C. D., Cordon-Cardo, C., and Gilboa, E., The role of IL-2 secreted from genetically modified tumor cells in the establishment of antitumor immunity. *J. Immunol.* **152**, 2324–2332, 1994.

25. Eggert, A. A., Schreurs, M. W., Boerman, O. C., Oyen, W. J., de Boer, A. J., Punt, C. J., Figdor, C. G., and Adema, G. J., Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res.* **59**, 3340–3345, 1999.

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Progress in reproductive biotechnology in swine.

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Mariensee (FAL), Neustadt, Germany.

This article summarizes recent progress in reproductive biotechnology in
swine with special reference to in vitro production of embryos, generation of
identical multiples, and transgenic pigs useful for xenotransplantation. In
vitro production (in vitro maturation, in vitro fertilization, and in vitro
culture) of viable porcine embryos is possible, although with much lower
success rates than in cattle. The main problems are insufficient cytoplasmic
maturation of porcine oocytes, a high proportion of polyspermic fertilization
and a low proportion of blastocysts that, in addition, are characterized by a
low number of cells, hampering their development in vivo upon transfer to
recipients. Microsurgical bisection of morula and blastocyst stage embryos
leads to a 2 to 3% monozygotic twinning rate of the transferred
demiembryos, which is similar to that in rabbits and mice but considerably
lower than in ruminants. It was found that with decreasing quality an
increasing proportion of demi-embryos did not possess an inner cell mass.
Porcine individual blastomeres derived from 4- and 8-cell embryos can be
cultured in defined medium to the blastocyst stage. Leukemia inhibitory
factor has been shown to be effective at defined embryonic stages and
supports the formation of the inner cell mass in cultured isolated
blastomeres in a concentration-dependent manner. For maintaining
pregnancies with micromanipulated porcine embryos, it is not necessary to
transfer extraordinarily high numbers of embryos. Porcine nuclear transfer is
still struggling from the inefficiency of producing normally functioning
blastocysts. Blastomeres, blastocyst-derived cells, fibroblasts and granulosa
cells have been employed as donor cells in porcine nuclear transfer and have
yielded blastocysts. Recently, the generation of the first piglets from somatic
cell nuclear transfer has been achieved. DNA-microinjection into pronuclei
of porcine zygotes has reliably resulted in the generation of transgenic pigs,
which have special importance for the production of valuable

pharmaceutical proteins in milk and xenotransplantation. It has been demonstrated that by expression of human complement regulatory proteins in transgenic pigs the hyperacute rejection response occurring after xenotransplantation can be overcome in a clinically relevant manner. Although biotechnological procedures in swine have recently undergone tremendous progress, the development is still lagging behind that in cattle and sheep. With regard to genetic engineering, considerable progress will originate from the possibility of employing homologous recombination in somatic cell lines and their subsequent use in nuclear transfer. In combination with the increasing knowledge in gene sequences this will allow in the foreseeable future widespread use in the pig industry either for agricultural or biomedical purposes.

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